Probing sensory perception in multiple dimensions: The development of real-world environment and optical tracking methods for head-fixed mice.

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To my parents and many intellectual heroes who encouraged me to fumble around in the glorious pursuit of knowledge.

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Abstract

Natural behavior occurs in multiple sensory and motor modalities and is dependent on sensory feedback that constantly adjusts behavior. To investigate the underlying neuronal correlates of natural behavior, it is useful to have access to state-of-the-art recording equipment that frequently requires head-fixation. This limitation has been addressed with various approaches such as virtual reality with air ball or treadmill systems. However, these systems have several disadvantages. Here we developed a novel tool, the Air-Track system, an easy to build, head-fixed behavioral environment that requires only minimal computational processing.

The Air-Track is a lightweight, physical maze floating on an air table that has all the properties of the “real” world, including multiple sensory modalities tightly coupled to motor actions. To test this system, we trained mice in Go/No-Go and two-alternative forced choice tasks. A custom-controlled camera system monitored animal location, and generated data that could be used to calculate reaction times in the visual and somatosensory discrimination tasks. To track the motion of the Air Track system we developed a “Pixy” tracking system based on an off-the-shelf camera system (Pixy). We then expanded the development into a generalized and automated optical method for real-time and post-hoc tracking of mice motor behavior in both head-fixed and freely moving conditions.

Air-Track and Pixy-Tracking systems are convenient “one-size-fits-all” solutions that facilitate the combination of quantitative natural behavior with virtually any system for monitoring or manipulating brain activity in a neuroscience laboratory.

Zusammenfassung

Natürliches Verhalten findet in diversen sensorischen und motorischen Modalitäten statt, und hängt vom sensorischen Feedback ab, welches das Verhalten kontinuierlich anpasst. Um die zu Grunde liegenden neuronalen Korrelate natürlichen Verhaltens untersuchen zu können, ist die Nutzung moderner Aufnahmetechniken notwendig, die oft die Kopffixierung des Tieres erfordern. Diese Einschränkung wurde mit verschiedenen Methoden angegangen, unter anderem mit virtueller Realität in Kombination mit einem luftgelagerten Ball oder Laufradsystemen. Diese Systeme haben jedoch zahlreiche Nachteile. Wir haben das Air-Track-System entwickelt, eine neue Methode für eine leicht zu bauende und nur minimale Computerverarbeitung erfordernde Verhaltensumgebung.

Der Air-Track ist ein leichtgewichtiges physisches Labyrinth, das auf einem Lufttisch schwebt und alle Eigenschaften der "echten" Welt hat, einschließlich mehrerer sensorischer Modalitäten, die eng an die motorischen Handlungen gekoppelt sind. Um dieses System zu testen, trainierten wir Mäuse in Go/No-Go- und two-alternative forced choice-Aufgaben. Mäuse wählten Arme und unterschieden. Ein Kamerasystem mit eigens entwickelter Kontrolle zeichnete die Position des Tieres auf und generierte Daten, die zur Berechnung von Reaktionszeiten in den visuellen und somatosensorischen Unterscheidungsaufgaben verwendet werden konnten. Um die Bewegung des Air-Track-Systems aufzuzeichnen, entwickelten wir ein "Pixy"-System zur Bewegungsverfolgung. Wir erweiterten die Entwicklung dann zu einer allgemeinen und automatisierten optischen Methode für die Echtzeit- ebenso wie die nachträgliche Verfolgung der Mausbewegungen, und zwar sowohl für kopffixierte als auch frei bewegliche Tiere.

Das Air-Track-System und die Pixy-Bewegungsverfolgung sind zweckdienliche Einheitslösungen, die die Kombination von quantitativem natürlichen Verhalten mit nahezu jedem System zur Aufzeichnung und Manipulation der Hirnaktivität in einem Hirnforschungs-Labor ermöglichen.

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Thesis outline

Chapter 1 briefly introduces the status quo of behavioral neuroscience research with particular focus on the use of virtual reality systems in rodents' behavior to study brain functions.

Chapter 2 describes the technical development of the Air-Track system and the behavioral experiments used to verify its use on head-fixed mice experiments.

Chapter 3 describes the technical development of Pixy-Tracking system and the behavioral experiments used to verify its use on head-fixed and freely moving mice.

Chapter 4 discusses the main findings of the Air-Track and Pixy-Tracking systems with focus on future directions and ethical aspects in behavioral electrophysiology research.

Chapter 1: Introduction

The brain can be investigated on many levels from molecular to cellular and neural networks, right up to behavioral and imaging experiments in humans. Behavioral neuroscience in animals is considered an important pillar within this spectrum of approaches because it provides the opportunity to combine system-wide observations with detailed cellular and network approaches that require intervention (i.e. operations for invasive recordings that are impossible in humans) (Devor et al. 2013). The main goal of system neuroscience is to understand how a distributed network of neurons act in concert to produce behavior. In order to achieve this goal, it is important to have not only quantitative measure of neural activity but also systematically track the relationship between neural activity and behavior becomes necessary.

1.1. Behavioral neuroscience

A proper measurement of behavior requires rigorous description of subtle observable muscular output of an organism in the external world. In general, behavior of any organism has three main features that could be utilized for quantitative measurements. 1) It is relational to the animal's external world describing the contextual interaction between the animal and its environment. 2) It is dynamic and continuous process manifested in space and time that can be parsed into discrete events. 3) It is complex, variable and has multiple dimensions that often engages different modalities, behavioral states, and even social interactions during goal-directed behavior (Gomez-Marin et al. 2014).

Historically, two mainstream approaches have been recognized for the study of behavior. Ethologists initiated the first stream by studying behavior in the animal's natural environment. They tried to understand evolutionary origin of natural behavior and the correlations between stereotypically similar

behaviors across species. They coined the term “Ethome” as the behavioral unit that includes small behavioral patterns interconnecting similar behaviors within a species (Tinbergen 1977). While ethologists study animal behavior in natural setting, the second approach, adapted by psychologists and physiologists, have attempted to study behavior in more controlled level in the laboratory conditions. This approach generally focuses on a stimulus and response behavior where stimuli, action and outcome of the animal can be well controlled and measured, as was done in the classical and operant conditioning experiments (Konorski 1968).

In systems neuroscience, scientists tend to reduce the complexity of the behavior, to make it possible to relate neural activity to a distinct and well-controlled aspect of behavior. Although reducing behavioral complexity simplifies the study of the neuronal responses, it compromises the mutli-dimensionality of the functional relationship between large-scale distributed neuronal networks and behavior. Reducing behavioral complexity effectively minimizes the active sensing dimension of behavior which requires multimodal interaction between the animal and its environment (Gomez-Marin and Mainen 2016). Designing a behavioral electrophysiology experiment has always faced interconnected challenge of constraining the animal to a degree that allows proper neuronal investigation while maintaining a high degree of behavioral measurements with multiple levels of description (**Figure 1**) (Gomez-Marin et al. 2014).

With the advent of modern technological development for recording from the brain (using electrical and optical methods), there has been a recent flourishing of approaches for understanding how neurons operate in the behaving animal. However, many of these approaches require near-absolute stability, meaning (in most cases) that these recordings require the head of the animal to be completely still. Head-fixation raises interesting problems for combining it with meaningful behavior. There are many different solutions to this problem but they usually take one of two broad strategies: 1) reducing the behavior such that it can be performed in an animal that is fixed to the

recording apparatus. 2) Using strategies such as virtual reality combined with head-fixation to allow more complex or even near-realistic behavior.

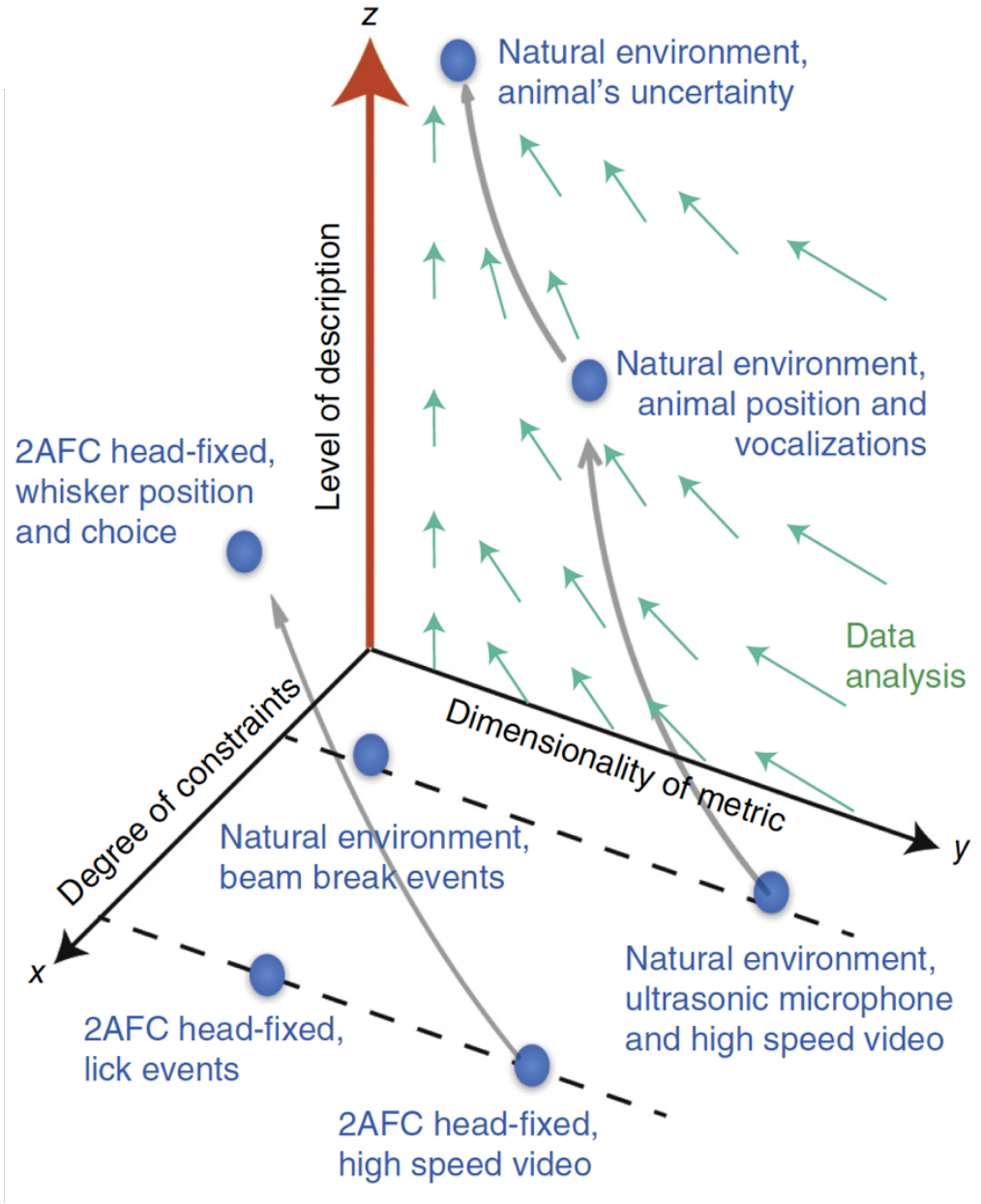

Figure 1. Conceptual depiction of behavioral science space axes

In the depiction of behavioral space, the x-axis is experimental constraints, the y-axis is measurement dimensionality, and the z-axis is the level of description. The degree of constraints (low by ethologists, high by physiologists) affects the behavioral dimensionality. The introduction of computers and information technology in behavioral sciences have bridged the gap between setting a degree of constrain for recording and having a relatively high degree of dimensionality and description. For instance, animal behavior in naturalistic environment can be constrained by setting a 2AFC task in a head-fixed preparation. With such high constraints, combining a high-speed camera to analyze whisker movement, reaction time and animal decisions increases the dimensionality of data. Adapted with permissions from Gomez-Marin and colleagues (Gomez-Marin et al. 2014).

In this study, we present a system that extends the 2nd approach by designing a “real-world” behavioral apparatus that itself moves around the animal under the control of the mouse itself. This system allows us to investigate multiple sensory modalities simultaneously in naturalistic conditions. In addition, we have developed a real-time tracking system that allows us to quantitatively monitor the behavior on both the macro and micro level. The use of both systems expands a wide range of experimental possibilities across the three axis of the behavioral sciences space simultaneously by enabling high-resolution neural and behavioral data in naturalistic environment (**Figure 1**).

1.2. Sensory modalities in rodents

The brain organization of the rodents and primates has a lot of basic similarities. The brain anatomy shares a neocortical structure that shows a similar plan and division for multiple areas dedicated for defined sensory modality such as visual and somatosensory cortex (Krubitzer 2007). However, nocturnal animals like rodents rely heavily on olfaction and somatosensation to make reliable and subtle decisions (Kepecs et al. 2008). This is also reflected in the relative amount of brain tissue devoted to the olfactory blubs and the so-called barrel cortex responsible for whisker sensation. Although rodents are less dependent on the use of their vision in behavior, recent studies have showed rodents' ability to do complex behavior using only their vision such as spatial navigation (Holscher et al. 2005). Before discussing different methods used to study neural basis of behavior in rodents we briefly describe rodent's different sensory systems.

1.2.1. Visual system

Because rodents are nocturnal animals, they generally rely on their somatosensory and olfactory modalities to navigate the environment. However, studies have shown that vision still contributes to the animals navigation, as it extracts information about distal and proximal feature of the environment. Unlike higher primates, rodents have relatively poor vision with only two color receptors, blue/UV short wavelength cones and medium wavelength cones (Jacobs et al. 1991; Szel and Rohlich 1992). Rodent vision is limited to long wavelengths such as red color, but they still can see in the short wavelengths of the ultraviolet range (Szel and Rohlich 1992). In addition, rodents have poor color recognition and visual acuity (Prusky et al. 2000). With only 1% cone receptors on their retina, this limits their ability to detect different colors. Rodents have lower resolution (blurry) with a visual acuity thirty times lower than human visual acuity (Jacobs et al. 2001). Concomitantly, rodents have a larger field of view, extending horizontally to 300˚ (Hughes 1979).

1.2.2. Somatosensory system

During navigation, rodents predominantly use their somatosensory system, particularly, their whisker system, to navigate the environment. Through a process called active whisking, where animals sweep their whiskers forward and backward, rodents collect sensory information about walls, position of objects, their size, texture and geometrical features (Berg and Kleinfeld 2003b; Diamond et al. 2008). Active whisking improves the discriminability by increasing the acuity, resolving spatial offset smaller than intervibrissal spacing (Knutsen et al. 2006).

Each whisker is attached to the facial pad through a follicle that provides the whisker with both the sensory and motor enervation (**Figure 2A**). Follicles receive enervation from the trigeminal ganglion consisting of 200 cells that convert mechanical input into action potential. The trigeminal ganglion conveys information through an afferent synapse to the trigeminal nuclei in the brain stem. The trigeminal nuclei in turn projects to the thalamus through two parallel pathways, the lemniscal pathway targets VPM and the extra-lemniscal pathway targets POM, before it continues to the barrel field in somatosensory cortex (**Figure 2B**). Macrovibrissae, large whiskers, are arranged in grid made of 5 rows labeled from A to E and numbered as arches so each individual whiskers can be identified as a coordinate of its row and

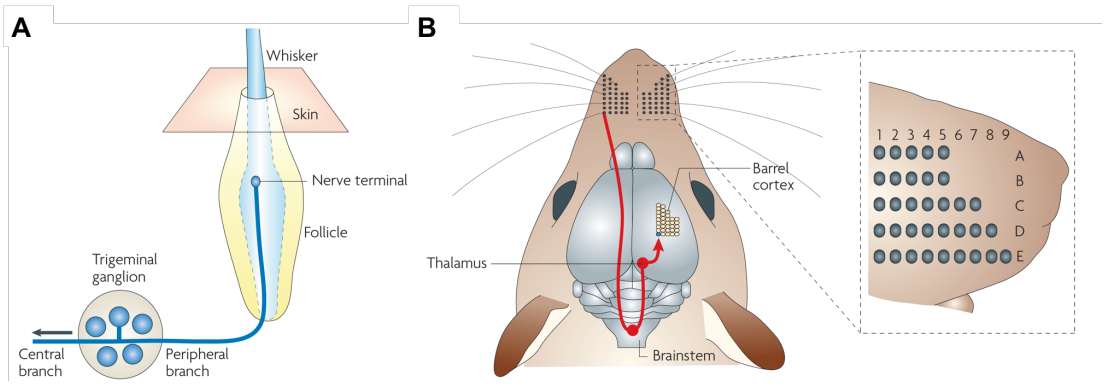

Figure 2. Schematic of the whisker sensory pathway

A Schematic for whisker sensorimotor innervation through follicle to the trigeminal ganglion. **B** schematic for two-dimensional grid of vibrissa of the whisker pad innervate through the thalamic nucleuses to a corresponding grid-like barrel field in layer 4 of primary somatosensory cortex, adapted with permissions from (Diamond et al. 2008).

arc (e.g. B2) (Clarke and Bowsher 1962; Deschenes et al. 2005; Woolsey and Van der Loos 1970).

The organization of the whisker pad is replicated in the anatomy of the barrel region in somatosensory cortex so that each whisker has an ascending pathway connected to a barrel. Studies have shown that rodents use their whiskers predominantly in various cognitive tasks such as gap crossing, object localization, texture discrimination and to determine the direction of air flow (Diamond et al. 2008; Yu et al. 2016).

1.2.3. Olfactory system

Olfaction is a sensory modality that has a fundamental importance in rodents characterized with high accuracy and reliability in providing information. In nature, most odors consist of a mixture of chemical compounds that bind with different affinities to any of the olfactory bulb 1200 receptors (Mombaerts 2004). Each olfactory receptor type is expressed in a specific neuron. The spatiotemporal firing pattern of these 1200 olfactory neurons defines the acuity in discriminating between different mixtures of odors (Spors and Grinvald 2002). During navigation, rodents synchronize sniffing with whisking to navigate objects and localize food and danger in their environment (Rajan et al. 2006).

1.2.4. Auditory system

Rodents have an extremely large audible frequency range compared to humans of between 200 Hz to 90 kHz (Kelly and Masterton 1977). They are able to hear well in the ultrasound range and vocalize between 20 kHz to 50 kHz (Thomas et al. 1983). On the other hand, they have poor angular auditory resolution that is limited to 11.1˚ due to the small spacing between their ears (Kavanagh and Kelly 1992).

1.3. History of virtual reality development

The concept of virtual reality has existed in the artistic traditions since ancient civilization through the creation of art works that appears to expose a physical space, so called mimesis. The development of illusory visual environment was classically static and limited to artwork such as painting until the twentieth century, where dynamic virtual environments started to emerge. In 1930, Edward Link developed a commercial flight simulator so called the "Link trainer" (Jeon 2015). This system was used to train pilots in safer conditions before flying with a military aircraft. The Link trainer consisted of motors connected to a rudder and steering column to manipulate pitch and roll and simulate turbulences experienced in military flights.

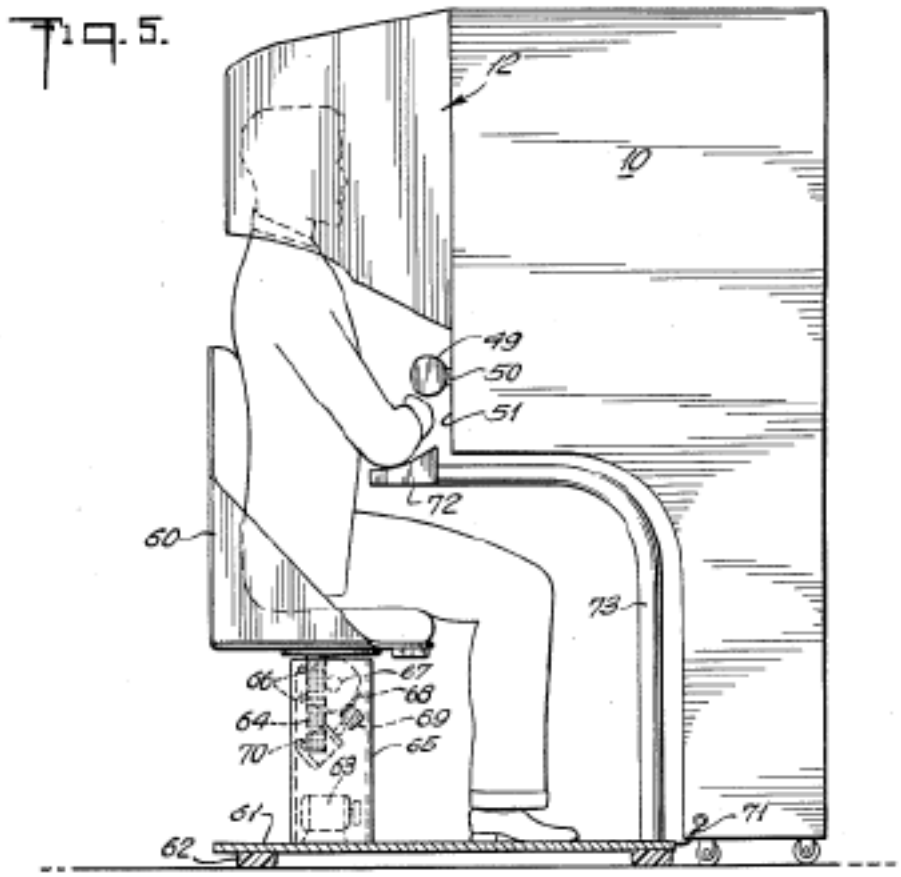

Figure 3. The Sensorama.

A side view of experience theatre comprises developed my Morton Heilig at 1957. Adapted from Morton Heilig's patent (The Sensorama) (1962).

In 1950's, Morton Heilig developed the "Sensorama" also called Experience Theater (**Figure 3**). Sensorama was an immersive multimodal virtual reality with stereoscopic 2D display, stereo speakers, fans and olfactometer. In "Sensorama", Heilig created six movies integrated different modalities to simulate real experience (Boese 2007). The emergence of digital computing in the 1950s witnessed acceleration in the nature of virtual reality technologies. These technological developments focused on constructing a visual virtual world rather than creating a multimodal reality.

Currently, virtual reality is widely used in cognitive neuroscience in behavioral and functional imaging studies (Ekstrom et al. 2003; Moffat et al. 2006; Tarr and Warren 2002). It has also shown a great promise in clinical applications when integrated with conventional medical approaches for the treatment of stroke and paraplegia (Donati et al. 2016; Laver et al. 2015). In the last 5 years, virtual reality technologies have made their way into the consumer marketplace. Using an immersive visual-motor virtual reality, different types of commercial gadgets and headsets are used in gaming and entertainment industry.

1.4. Virtual reality in animals

The design of a virtual reality for an animal involves the development of a closed loop system in which a dynamic simulation of the animal's environment is constructed, controlled and updated based on animal motor action (Benda et al. 2007). The majority of virtual systems for animals exploit computer-controlled visual displays to generate a virtual visual stimulus that, changes the visual stream according to the readout of animal movement (**Figure 4**). The advantages of virtual reality are many. First and foremost, VR provides a parametric interaction between the animal and the environment that can be investigated on the neuronal level. This can be done while the animal is stationary allowing the behavior to be performed alongside sophisticated equipment. The main challenge is to create a quasi-naturalistic stimulus and efficiently map the animal's movement trajectory to a corresponding change in the virtual world (Dombeck and Reiser 2012).

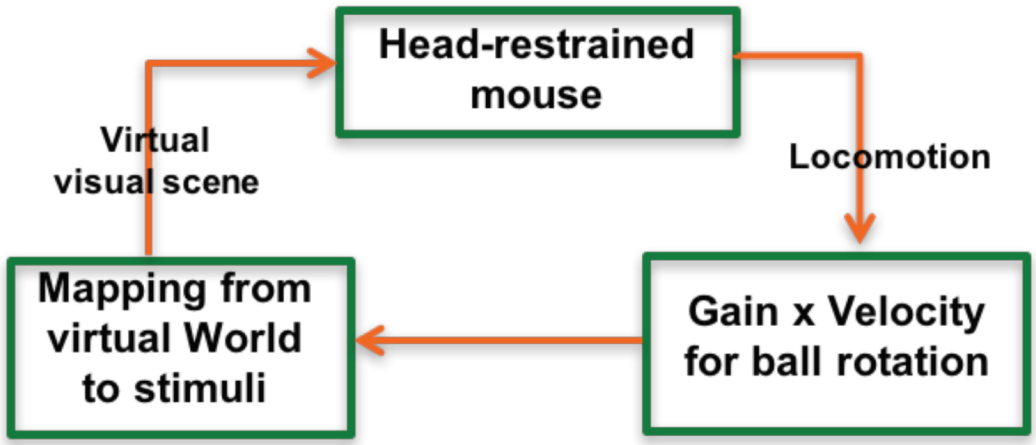

Figure 4. Conceptual sketch of constructing a closed–loop system for rodents' virtual reality system.

In the last decade, several studies have investigated neural activity in behaving animals using VR systems. Despite the complexity and the expense of using VR system in comparison to classical behavioral system, VR systems have four main advantages that encourage a lot of system neuroscientists to use in their investigation. 1) VR systems enable precise control over the simulated stimulus/environment where both the timing presentation and the the complexity of the stimulus can be tuned over trials/sessions. 2) Most VR

approaches in animals utilize head-fixed preparations allowing a wide range of sophisticated recording and imaging techniques that require mechanical stability, such as two-photon imaging and whole-cell recording and functional magnetic resonance imaging. 3) Animal restraint allows precise monitoring of animal behavior during interaction with the environment that is hard to track in freely moving behavior. 4) By deliberately introducing unexpected perturbations, it is possible to investigate how the brain codes, copes, and updates information in the face of mismatches between behavior and the change in the environment.

1.5. Technical elements for developing a VR system

The construction of a VR system requires combining the following three experimental and technological methods:

1.5.1. Tethering the animal

Animal restraint is a classical experimental technique that facilitates neural recording from the brain while allowing enough freedom for the animal to move in response to the stimulus (Devor et al. 2013; Kuhn et al. 2008; O'Connor et al. 2010; Poulet and Petersen 2008). In virtual reality, this methodology enables high-resolution neural recording techniques such as two-photon imaging and whole-cell recording combined with complex quasi-natural behavior (Harvey et al. 2009; Maimon et al. 2010). Some of the earliest experiments that used animal restraint in the course of studying animal behavior were on vineyard snails in 1930's. Jakob von Uexküll reported the study of the snail's motor reaction time by tethering the snail's

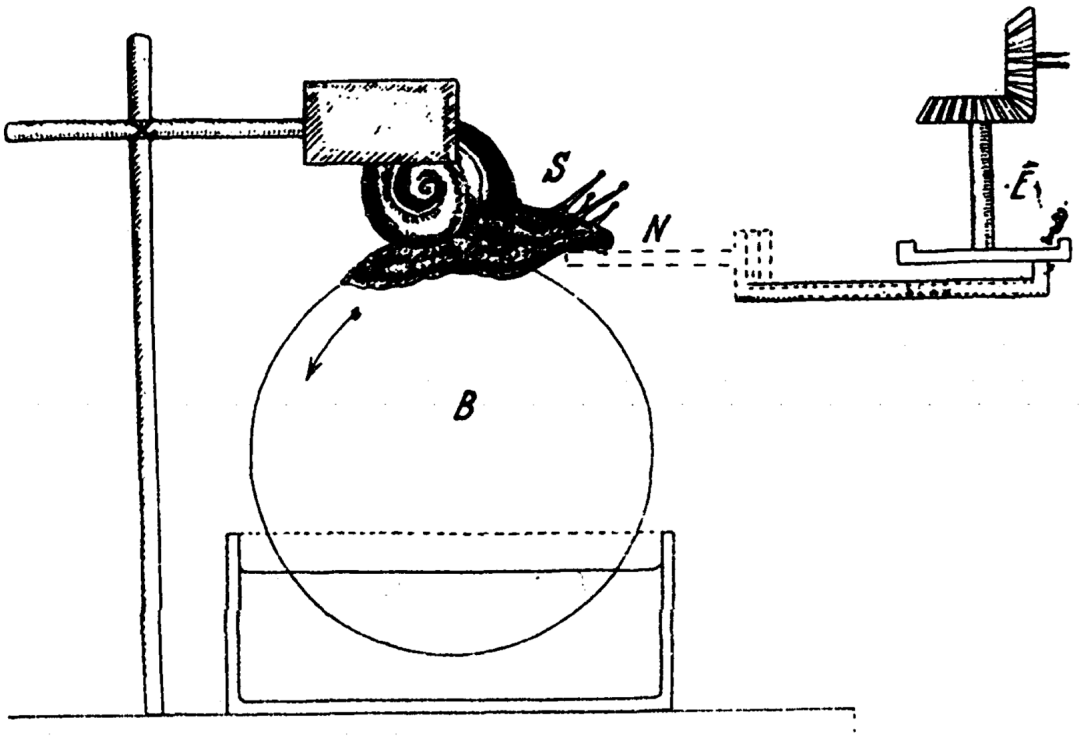

Figure 5. Uexküll's shell-fixed snail treadmill

The snail shell is held in place as the snail moves a rubber ball floating in water. The movement of the ball is instead by a cue. Adapted with permission from Jakob Von Uexküll (Von Uexküll 1992).

shell between brackets while placing the snail in a rubber ball carried by water and sliding without friction (**Figure 5**) (Von Uexküll 1992).

Later, a series of noteworthy experiments were performed at Max Plank Institute for Biological Cybernetics in Tubingen using a similar tether to hold the fly while allowing the wings to beat freely (Gotz 1968). A similar method was also used in zebrafish and *C. elegans* by fixing the head in agarose while allowing the rest of their body to move freely in fluid (Chronis et al. 2007; Ritter et al. 2001). In rodents, body or head restraint have been used while the animal moves either on a linear treadmill, or a spherical treadmill (air-ball) (Dombeck et al. 2007).

1.5.2. Tracking animal movement

Adequate Tracking for animal movement is critical to close the loop between animal interactions with the simulated environment. Restraining the animal makes it possible to track the relevant motion of the animal and to generate a real-time readout of animal movement that can be used as a feedback signal to trigger the update in the simulated environment. In insects, motor behavior can be reported directly by measuring the wing beats of the tethered fly using an electromechanical torque meter or optical wing tracking (**Figure 6A**) (Gotz 1968).

Most other approaches achieved indirect report of animal movement by using a low friction, air-supported smooth spherical treadmill equipped with motion sensor and two side wheels (Dahmen 1980). The role of the side wheels was to restrict the movement of the sphere and encodes the movement optically. This system was first introduced in walking insects to detect the direction and orientations of movement. Much later, a bigger and lightweight Styrofoam ball were adapted for rodents studies (Holscher et al. 2005). The ball is airlifted with a constant pressure of air stream directed underneath the ball creating a frictionless air-cushion underneath the sphere to float it of the ground (**Figure 6B**).

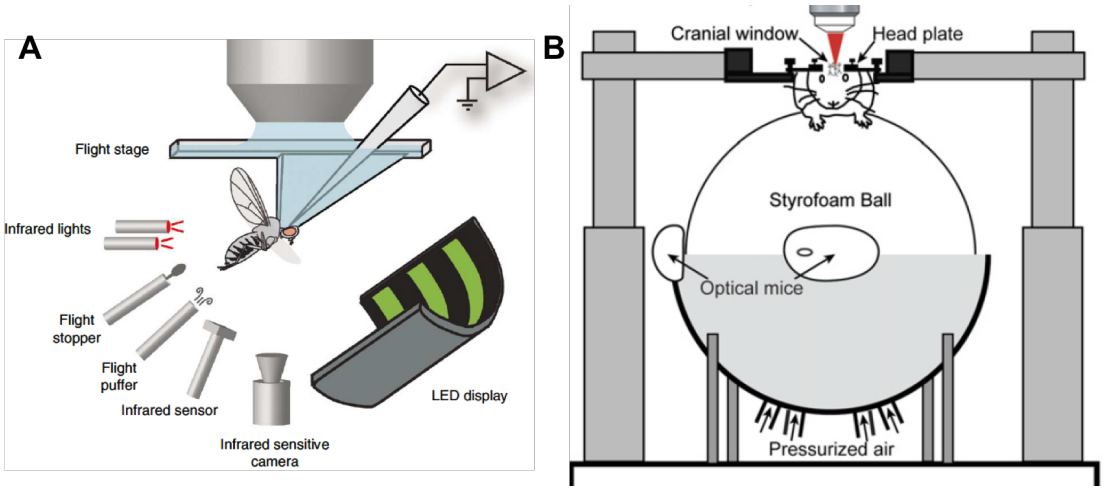

Figure 6. Different methods for reporting animal movement across species.

A Schematic of fly behavior in virtual reality; wing beat amplitude and head movements are measured using an infrared sensor and camera. **B** Schematic of mouse on an air-ball during 2P imaging from motor cortex first reported by Dombeck and colleagues, adapted with permissions from previous studies (Dombeck et al. 2007; Maimon et al. 2010)

Despite its wide use for studying behavior in a variety of species, the air-ball system has some key limitations. 1) There is a little motor flexibility in virtual reality systems: in air-ball based virtual realities, motor readout is processed for 2D, yaw movement around the vertical axes and linear movement around the horizontal axes; in treadmill based VR systems are considered 1D virtual reality in which only movement is around a single axes is processed for update of the virtual environment for either forward or backward movement. 2) Larger animals, like mice and rats, require a larger ball which often increase the bulk of the apparatus and reduces the flexibility of the system for a variety of imaging and physiology approaches. 3) Rodents with big weight require an increase in the airflow supporting the sphere, which increases that acoustic noise of system making it incompatible with particular auditory experiments.

1.5.3. Constructing the virtual world

Simulating the real world is considered the trickiest aspect of virtual reality, partely because it requires choosing the proper type of sensory stimuli to simulate reality. These stimuli vary for each species, and require

parameterization. Even after selecting the appropriate stimuli, virtual environments are often imperfect However, the virtual construction would still be efficient if the animal can envisage the stimuli and interact with the environment in a proper manner to perform behavioral task such as navigation or decision making. The vast majority of animal VR systems are based on visual-created virtual environment. Lately, there have been new developments in VR systems based on tactile or auditory modalities.

1.5.3.1. Visual virtual reality

Visual virtual reality is the most popular VR system used in rodent experiments. To construct an efficient visual-created virtual stimulus for rodents, VR systems use large panoramic visual displays with low illumination and resolution to match the optical properties of rodents visual systems characterized by low acuity and by collecting large amount of light (**Figure 7**) (Holscher et al. 2005). A panoramic visual view can be either created by using panoramic displays or combining a projector with amplification mirror (Dombeck et al. 2010; Harvey et al. 2009). A crucial factor for constructing an efficient and reliable VR system for rodents is motor readout from air-ball movement. This movement is used to update the visual scene in a closed loop fashion.

In rodents, the average reaction time for motor output is around 100 ms, thus it is possible to easily use a graphic display software with a 100 ms

Figure 7. First virtual reality system for rodents

First implementation of virtual reality for harnessed behaving rats, adapted with permission from Holscher and collogues (Holscher et al. 2005).

update rate (Mauk and Buonomano 2004). In flies, visual acuity is lower than rodents, but flies sample their visual space at higher rates (Reiser and Dickinson 2010). Thus, for a faster motor output 80 ms, LED displays are used to provide an efficient remapping between motor feedback and corresponding change in LED display. In zebrafish, swifter sets of LED displays are used to couple with larva zebrafish reaction time around 10s of ms (McElligott and O'Malley D 2005).

1.5.3.2. Tactile virtual reality

Rodents predominantly using their whiskers in navigating dark burrow and hunting for food (Diamond et al. 2008). To introduce tactile element to virtual reality systems, recent methodological study has transformed the commonly used visual-created VR system into a tactile virtual environment for head-fixed mice (**Figure 8**) (Sofroniew et al. 2014). In this study, mice were fixed on the top of a ball, while surrounded by two movable walls on the both sides of the animal instead of the display. In a closed loop design the movement of the wall was determined based on two factors. 1) A computer program that dictated by the experimentalist to develop a virtual path during navigation. 2) Mapping the feedback generated by the mouse movement on

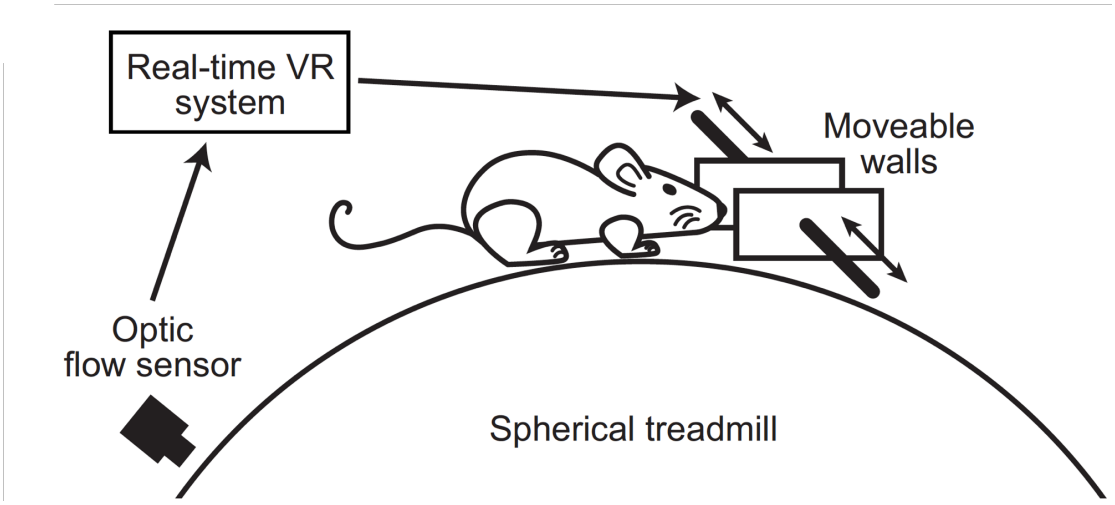

Figure 8. Tactile virtual reality for head-fixed mice.

A schematic side-view of the mouse on air-ball. The movement of the mouse on air-ball was measured using two optical sensor to update the position of two side-walls, adapted from Sofroniew and collogues (Sofroniew et al. 2014).

the ball to the walls based on animal movement. The system used whisker guided locomotion by simulating curvatures in the corridor, while keeping the virtual walls at specific but variable distance from each other to simulate a corridor.

We considered implementing a similar system for this study (indeed we managed to build a prototype), but it quickly became clear that there is a fundamental difference between the virtual simulation of visual stimuli and tactile stimuli. Namely, tactile stimuli must by their nature involve the touching of sensory apparatus (in this case the whiskers). This introduces many conceptual problems when simulating real-world objects like alternative corridors in a maze such as tactile flow and introducing angles and corners. We reasoned initially that the best way to simulate moving walls would be to move actual walls past the whisker apparatus. These technical prospects quickly lead to the idea of a “floating maze” and hence the Air-Track system.

1.6. Probing neural activity in VR environments

A growing number of studies have combined VR system with high precision recording techniques to probe neural activity. Although majority of these studies have used rodents for their promising genetic toolbox, the use of VR now expanded to study fly, fish and worms (Dombeck and Reiser 2012). The findings have contributed to our understanding of a variety of aspect of neural basis of cognitive processes that was hard to probe in classical behavioral electrophysiology settings.

1.6.1. Spatial navigation

As mentioned above, rodents, being nocturnal, are believed to rely more on their tactile and olfactory modalities to navigate their environment. Nevertheless, the majority of VR experiments, those that study spatial navigation for example, use a visual VR system because this is much easier to simulate for the reason just described. In these experiments even though visual input is solely used alone (without the other vestibular, tactile, proprioceptive, and acoustic input normally available in the real-world), rodents perform the spatial navigation tasks adequately (Holscher et al. 2005). It is therefore possible that rodents use visual cues mostly for navigating around object or with the help of cues from objects. Rodents could potentially recognize distant landmarks to orient themselves and perform spatial dependent behavioral tasks.

In VR studies, the virtual spaces of these mazes can extend to 10s of meters where different visual landmarks are presented to the animal for spatial navigation. Virtual cylinders can be attached to the ceiling with regular spacing between each other in 2D virtual environment (**Figure 9**) (Harvey et al. 2009). Also, the walls of virtual linear tracks or T-mazes could be made of different textures of grating to recognize by the mouse during spatial navigation. The first whole-cell recording from place cells in mice navigating a virtual corridor was made in 2009 (Harvey et al. 2009).

Later, the activity of place and grid cells were imaging during virtual navigation using two-photon microscopy (Dombeck et al. 2010). This technical

development enabled the understanding of the neural basis of a distinct cognitive function such as spatial navigation to the subcellular level.

Other Studies using VR have made it possible to study how inputs integrate within different compartments of place cells, such as somatic sub-threshold membrane potential and dendritic regenerative event. Recent study has shown that place cells tune its response to spatial stimuli in the virtual environment by integrating somatic and dendritic calcium events (Sheffield and Dombeck 2015).

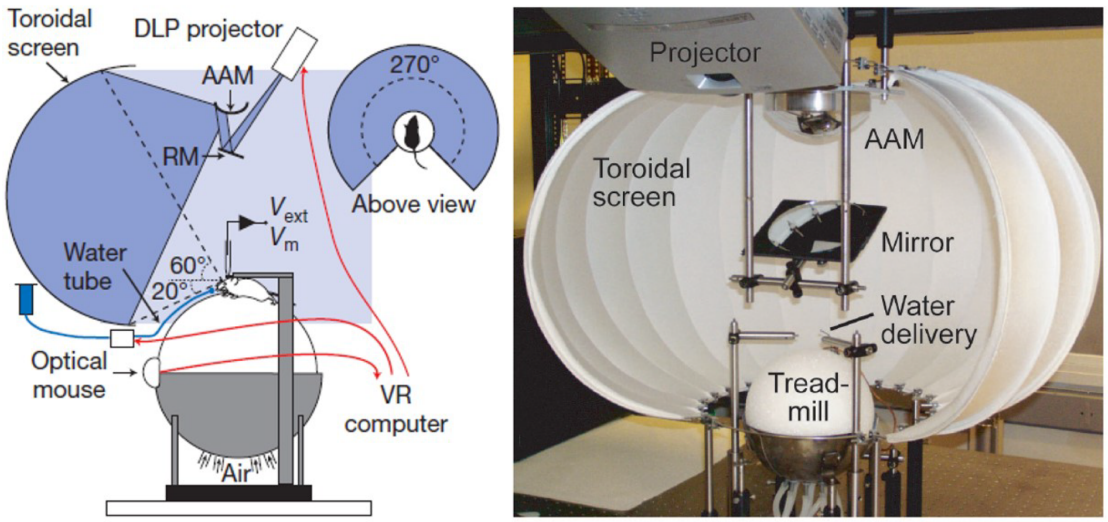

Figure 9. Virtual reality system for whole-cell recording in-vivo.

First implementation of whole-cell recording from place cells in virtual reality system in head-fixed behaving mice, adapted with permission from Harvey and colleagues (Harvey et al. 2009).

In other study, the ability to perturb animal behavior using optical manipulation was combined with calcium imaging (Rickgauer et al. 2014). The study shows that single place cell perturbation can indirectly change the tuning of other place cells in the local network, and consequently affects spatial navigation.

One popular behavioral paradigm for the study of hippocampus and spatial memory is to use a Morris water maze. However, the use of water maze hinders performing any imaging or electrophysiology recording. In a novel use of virtual reality, a combination of auditory and visual stimulus were

deployed to train rodents to navigate a virtual Morris water maze, in which electrophysiology experiments cannot be combined. In this audio-visual VR, the neural basis of spatial learning and memory can be studied in a virtual Morris water maze (**Figure 10**) (Cushman et al. 2013).

Another aspect of virtual navigation has been studied using a tactile virtual reality in which mice used their whiskers to navigate a virtual corridor using curving corridors (Sofroniew et al. 2014). As this behavior requires no training, this system could be used for understanding the role of somatosensory cues in tuning place cells activity during spatial navigational tasks using virtual tactile cues during virtual maze navigation.

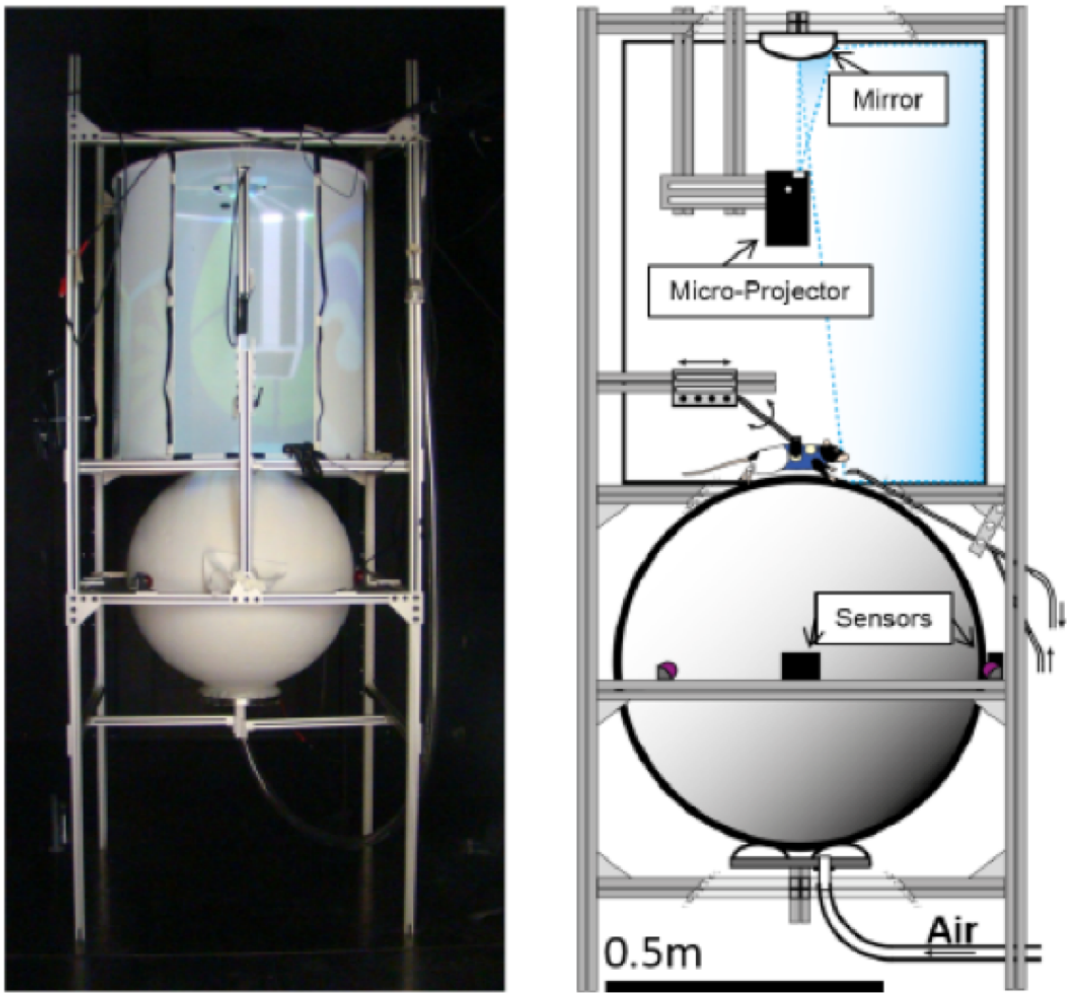

Figure 10. Multisensory visual-auditory virtual reality.

An implementation of multisensory virtual reality that mimic Morris water maze developed by Daniel Aharoni in Mehta's lab, adapted with permission from Cushman and colleagues (Cushman et al. 2013).

1.6.2. Sensory perception

The ability to introduce a precise and dynamic visual stimulus through a visual display has expanded the type of investigations that can utilize VR systems. It has become possible to take advantage of the interactive relationship between the dynamic nature of the virtual stimuli presented on the display and the treadmill/air-ball movement by the mouse. These type of experiments have shown that sensory processing in primary visual cortex is strongly modulated by locomotion.

The key advantage of visually-created virtual reality that it becomes possible to introduce mismatches between the actual and expected visual/motor feedback (Dombeck and Reiser 2012; Minderer et al. 2016). Studies using mismatches have focused on the neural basis of integrating motor and visual information both on the network and the cellular level (Keller et al. 2012; Niell and Stryker 2010; Rothman et al. 2015; Saleem et al. 2013). They created mismatches either by desynchronizing the ball and visual flow (open-loop mismatches) or modulating the mapping parameters between the ball movement and optical flow (closed-loop mismatches). A stunning result of these kinds of studies shows that single V1 neurons tune their responses by summing the weight of the visual flow and locomotion speed (Saleem et al. 2013).

Another study has investigated sensory processing in primary somatosensory cortex using a tactile virtual reality (**Figure 11**) (Sofroniew et al. 2015). In this study, S1 neurons were tuned to the distance between the mouse snout and the contralateral wall in the tactile virtual reality. The walls could even be mimicked via optogenetic stimulation for L4 neurons. A controlled optical stimulation can create an illusory wall effect and guide the mouse behavior to follow same trajectory of movement occurred during wall-tracking navigation.

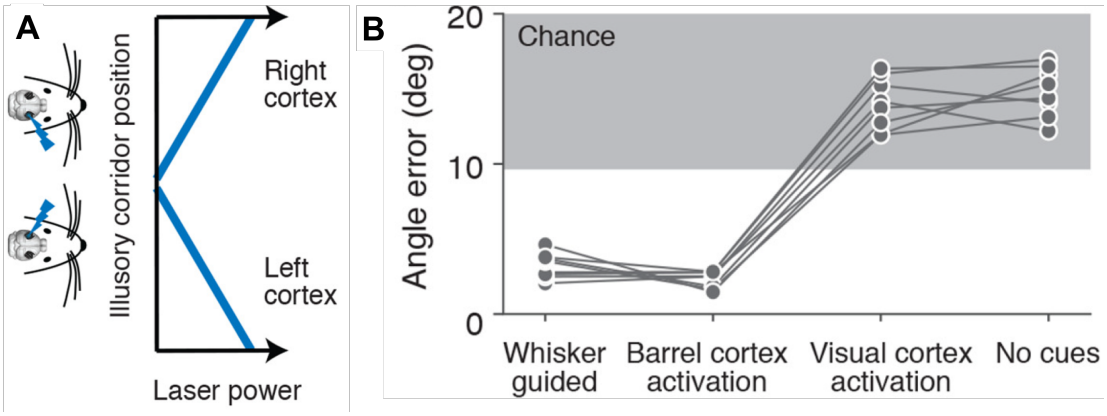

Figure 11. Photo-activation of layer 4 to guide locomotion in tactile virtual reality.

A schematic view of changing virtual corridor position generated by photo-activation showed on the left side. The degree of proximity is reflected on the intensity of the laser light on either of the two hemispheres to mimic whisker-wall contact. **B** angle error averaged along all trails for mouse navigating a curved corridor comparing mice with intact whiskers, barrel photo-activation, visual cortex photo-activation and without cues, adapted with permissions from Sofroniew and colloques (Sofroniew et al. 2015).

1.6.3. Decision-making

A recent study by Harvey and colleagues investigated the neural basis of decision-making in a VR environment (Harvey et al. 2012). In this study, mice were trained to navigate a T shaped maze, where they identified a visual cue at the start of a virtual linear track and they had to recall it at a later stage of this long linear track to make decision at the T-junction either left or right. As a working memory task the study focused on the neural interactions in posterior-parietal cortex that contribute to either one of the two decisions. The use of VR enabled studying the dynamics of neural population at different stages of behavior precisely by controlling the stimulus onset and timing between the two stimuli and decision-making stage.

Chapter 2: Air-Track: A real-world floating environment for active sensing in head-fixed mice

2.1. Introduction

Natural behaviors are complex and occur in multiple sensory-motor dimensions simultaneously. For example, a conversation between people or even a simple stroll down a corridor engages visual, auditory, tactile, kinesthetic, proprioceptive and olfactory senses. Accurate perception of these cues requires tight and reliable coupling to the motor system (Kleinfeld et al. 2006; Zagha et al. 2013). Mismatches between actions and sensory feedback profoundly disturb natural behavior (e.g. missing the “last step” while climbing stairs) (Keller et al. 2012). While natural behavior is usually performed during multimodal sensation/perception, the behaviors typically studied in lab settings are impoverished and often constrained to a single sensory or motor modality (Crochet and Petersen 2006). Advances in our understanding of the importance of natural, feedback-controlled behavior has triggered a gradual, but fundamental shift toward multimodal, multidimensional behavioral approaches.

Rodents are an ideal choice for neuroscientists interested in precise and invasive recording methodologies as they are relatively easy to train on simple tasks. Many of the experimental methods, however, require the head of the animal to remain stationary during the experiment. The early head-fixed rodent behavioral approaches were therefore based on reduced systems with only a single sensory modality, such as whisker movement (Bermejo et al. 1996; Crochet and Petersen 2006; Hentschke et al. 2006; Krupa et al. 2004; Sachdev et al. 2002; Welsh et al. 1995). The shift towards more complex, naturalistic, multimodal behavior began with awake mice head-fixed atop an air ball or a treadmill (Dombeck et al. 2009; Harvey et al. 2009; Poort et al. 2015). Importantly, various studies have reported that cortical responses are different when the animal is engaged in multidimensional behavioral tasks

(Dombeck et al. 2010; Harvey et al. 2012; Lenschow and Brecht 2015; Musall et al. 2014; Poort et al. 2015; Sofroniew et al. 2015). Just the act of walking on a treadmill changes visual and auditory responses (McGinley et al. 2015; Niell and Stryker 2010; Polack et al. 2013; Reimer et al. 2014; Saleem et al. 2013; Schneider et al. 2014; Sofroniew et al. 2015).

One advantage of the air ball and treadmill methods is that the movement of the mouse can be tracked and be used to control a virtual environment (Harvey et al. 2009; Holscher et al. 2005). For accurate correspondence between the mouse's movement and the virtual world, this environment is best represented using visual information usually in the form of 2D monitors in the visual field of the head-fixed animal. More recently, an equivalent somatosensory approach has been implemented (Sofroniew et al. 2014). This seemingly simple change in modality from visual to somatosensory presents a major advantage creating a natural tactile representation than virtual reality. However, it brings various problems in accurately matching the virtual representation to ordinary real-world situations, such as the representation of corners and optical/tactile flow. To be effective, virtual environments also require sophisticated software for tracking the animal and mapping these movements to the virtual world. This presents further difficulties estimating the perceptual experience of the animal, which is not necessarily intuitive for humans designing the mapping interface. For instance, place tuning of hippocampal neurons in rodents is different when they are in a virtual world as opposed to a real-world (Acharya et al. 2016; Aghajan et al. 2015). In practice, the qualitative perceptual experience of rodents in virtual reality systems is probably impossible to match with actual real-world experiences and, in any case, it is impossible to definitively demonstrate a correspondence between the subjective experience of the animal and the real-world.

Here, we present an alternative behavioral system as one solution to the problems of virtual reality approaches while retaining all the benefits of head-fixed experiments. Our system uses a real, physical environment that rests on a cushion of air and moves around the animal's body under the direct

control of the animal itself. The system, “Air-Track”, is based on the airlifted flat platform described by Kislin and colleagues (Kislin et al. 2014). We successfully used Air-Track to train head-fixed mice to perform a spatial orientation and multi-modal discrimination task within only two weeks.

2.2. Methods

Experiments were performed with approval of the local state authority in Berlin (LAGeSo) that is advised by the animal use ethics committee.

2.2.1. Air-Track components

The Air-track consisted of three essential custom-made components: First, an air-table which provided the air cushion; Second, a platform constructed of lightweight material for floating on air, that included the custom-designed maze with walls (**Figure 12A, B**); Third, a micro-controller system for tracking the position of the platform and controlling reward delivery (**Figure 12C**).

2.2.1.1. The air-table

Our solution used a transparent plexiglass box 20 X 24 X 3 cm, mounted on aluminum legs, forming a small table. The table had one intake port that was pressurized with air at 300 KPa (~45 psi) and small (1 mm) holes, spaced 8 mm apart, providing jets of pressurized air. The working surface of the table was 16 X 20 cm.

2.2.1.2. The platform

The circular platform (15 cm in diameter) was 3D printed. The base of the platform was 3 mm thick and this was sufficient to hold the platform floating steadily on a cushion of air. The platform attached atop the base, was shaped as a plus maze, with four lanes, each 5 cm long, 3 cm wide, and 3.5 cm in height and weighed 180 grams. In our design, the walls and terminal aperture of each lane could be modified; For textures, either smooth or with gratings etched with 2 mm spatial periods, and for aperture either 1 or 2 cm wide. Just above the platform, and outside the rotary axis of the maze, a white LED attached to a holder was used as a visual stimulus for choosing lanes. The LED was maintained in a constant state of either on or off based on the animal relative location in the maze. Similarly, a linear actuator used for positioning the lick spouts, was placed ~3 cm from the nose of the animal.

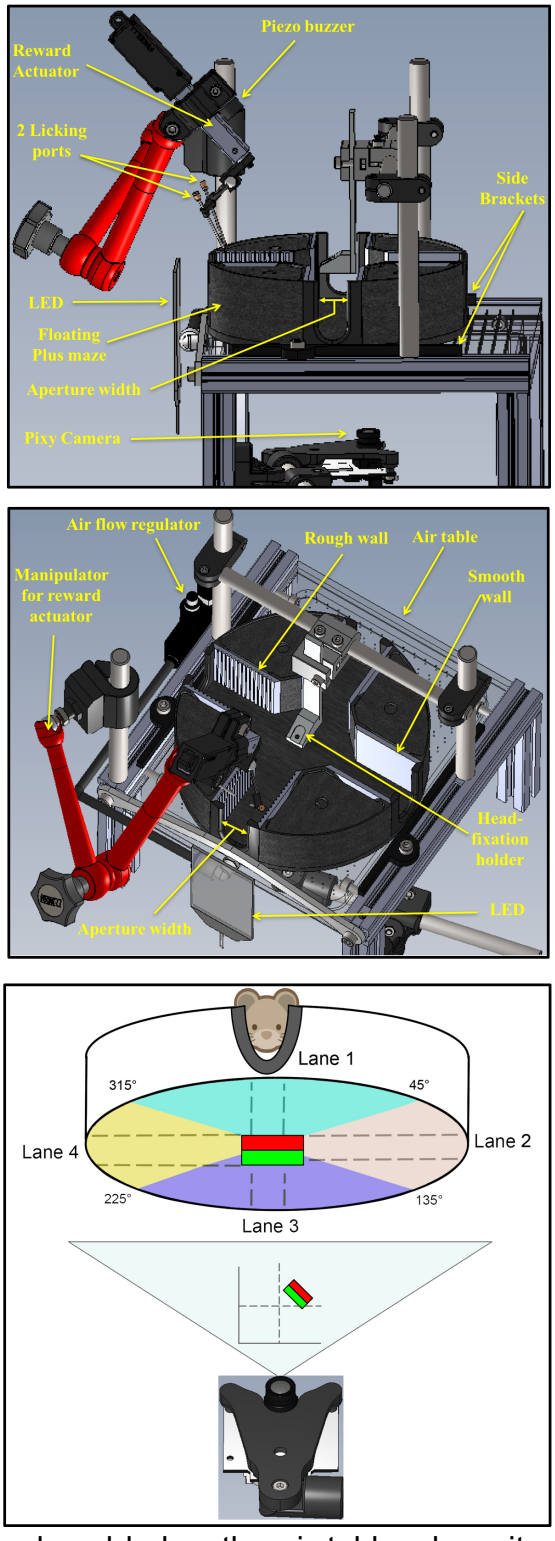

Figure 12. Air-Track setup design.

The setup enables closed-loop monitoring of behavior. Schematic of Air-Track design side-view **A** and top-view **B** The plus maze sits atop the plexiglass air table showing the LED for indicating correct lanes (on the left), the actuator for positioning the lick spouts (in black and red) also on the left, and the head post attachment in grey on the right. **C** A schematic of the Pixy camera and the red/green color tag under the Air-Track platform for position and orientation tracking. The camera has a 20 ms sampling resolution. Pixy camera uses color information from the maze color tag to determine the position of the animal in the maze.

The linear actuator advanced the dual lick spouts, connected to capacitive sensors, to the animal. When the animal arrived at the reward location in the maze, it could either passively obtain a reward irrespective of spout choice or performed a two-alternative forced choice (2AFC) between the two lick spouts.

2.2.1.3. Monitoring system

We chose an off-the-shelf approach to video tracking using a Pixy camera (CMUcam5 Image Sensor), placed below the air table where it could detect the rotation and position of a two-color mark glued to the bottom surface of the plus-maze (**Figure 12C**). This camera is unique in its ability to track colors, because it processes color information in real time on board, and reports the motion of the colored objects in x, y, and tilt angle with 50 fps precision. Therefore, when the Air-Track platform was moved the position and

orientation of the mouse within the maze was updated every 20 ms. The output was streamed to an Arduino-Uno micro-controller that processed Pixy camera inputs for animal location and also controlled the LED, the actuator that positioned the lick spouts, and the reward delivery solenoids (**Figure 13**). As the mouse rotated the maze, the camera detected its position and set the LED status to on/off based on the trial offset. The LED status for each lane was defined by a range of angles (i.e. ¼ circle sector) for each lane to be compared to the real-time data from the camera. With a given trial, the LED was turned off only while the mice faced the rewarding lane (**Figure 12C, Figure 14**).

2.2.2. Surgery

In preparation for head-fixation, adult 45 day old mice (C57BL/6) were anesthetized with Ketamine-Xylazine (90 / 10 mg / kg). A lightweight aluminum head post was attached using dental cement or Rely-X (3M ESPE, London, Ontario, Canada) (Andermann et al. 2013). Animals were monitored during recovery, and were given antibiotic (Enrofloxacin) and analgesics (Buprenorphine and Carpofen) during the recovery period.

2.2.3. Training paradigm

One week after surgery, mice were acclimatized to Air-Track in incremental 20 to 120 minute long sessions (**Figure 15**). They were placed in the plus maze, head-fixed and were periodically given 1ml/day of sugar-water (10% condensed milk with original sucrose concentration 45%) as a reward (Schwarz et al. 2010).

In the first 1-2 sessions (days) of training, we found it helpful if the experimenter was actively engaged with the platform and the animal, by gently nudging the platform, getting the animal habituated to the motion of the maze. During this active exploration phase, mice learned to rotate the maze, and to orient in the lanes, going forward and backward. In these sessions, a reward was given automatically when the animal had reached the end of any lane irrespective of any task parameters.

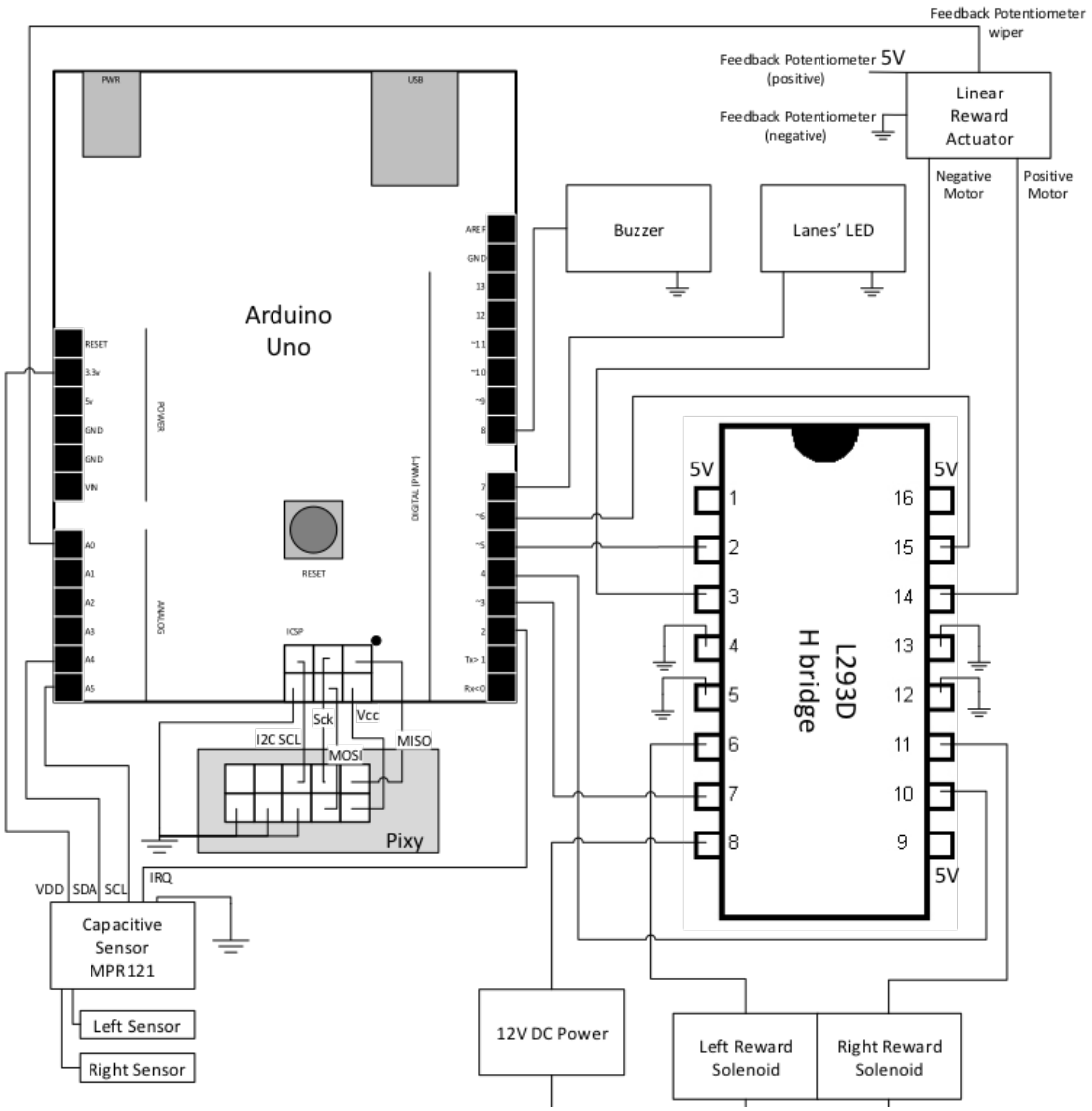

Figure 13. Schematic for Air-Track circuit.

Top left shows the schematic for Arduino-Uno connected through digital output to control a sound cue buzzer, LED, and H Bridge (L293D) to control linear reward actuator and two solenoids driven by a 12V DC power supply. The Pixy camera was connected to Arduino via ICSP port. The lick detectors (MPR121 module) were connected to Arduino via two analog and one digital inputs and powered with 3.3V.

In the 3rd-5th sessions, mice were acclimatized enough to propel the maze, and rotate it, without any need for experimenter intervention. During this active visual training phase, mice learned to choose lanes based on the presence or absence of a white LED / visual stimulus and collect the reward from a single lick of the reward spout. By the 6th to 8th session, the training paradigm shifted to a two-choice task, where visual stimuli still determined the correct lane, but the reward was only delivered from one of two lick spouts.

During this stage of passive tactile training, mice were given rewards automatically, without initially licking, at one of the two lick spouts based on the texture of the wall in order for them to passively learn associating each texture with one (left or right) of the two lick spouts.

From this point onwards, mice were trained for 4 to 6 sessions to discriminate either wall texture or aperture width. In this active tactile training phase, the mouse still had to choose a correct lane and actively discriminate either between two types of aperture width or wall textures. To obtain a reward, mice had to initiate licking the correct lick spout (decision determined by first lick).

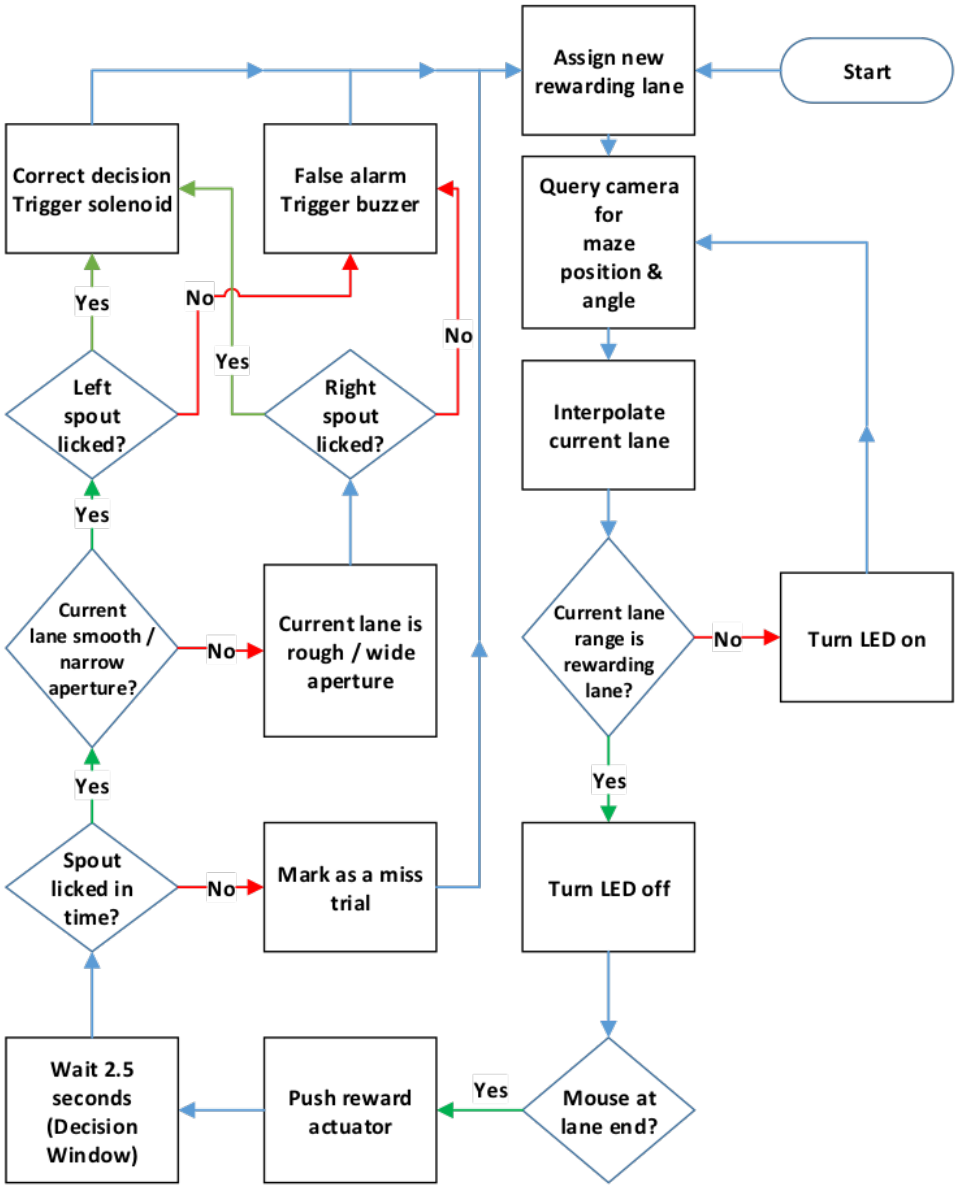

Figure 14. Flow chart of behavioral decisions in the Air-Track system.

The pixy camera keeps track of the color codes underneath the platform to report mouse position in relation to the platform. This information controls, accordingly, assigning a new lane, moving the linear reward actuator, the timing of visual and auditory cues, and reward delivery.

2.2.4. Behavioral training

Animals were water restricted (body weight stabilized >85% of initial weight) and conditioned to orient within the floating plus maze. The orientation of the lick spout was optimized for each mouse, in each session, to ensure that the mouse was positioned at an appropriate distance (~3 cm) from the lick spout. Animals were trained in near-total darkness, with a white light source directed under the non-transparent, black platform for the Pixy camera. The light was sufficient for the Pixy camera beneath the platform to track the position, but was not visible above the platform (**Supplementary Video 1**).

Each complete trial can be divided into 4 temporally distinct stages**.** The trial began with the mouse at the center of the plus maze (**Figure 16A, B).** In this phase of the trial, the mouse had to pay attention to the LED status to choose the correct lane. In order to find the correct lane, mice rotated the maze, clockwise or counter-clockwise. Over days mice developed an individual preference for the direction of platform rotation **(Supplementary Video 2)**.

The second stage was the choice of the correct lane by the mouse. This choice was reflected in the speed of rotation, and in the positioning of the head of the mouse with respect to the walls of the lane **(Supplementary Video 3)**.

The third stage was the entry into the lane. As the mouse moved forward, it touched with its whiskers the lane walls and the terminal aperture as the mouse approached the end of the lane. At this stage, the linear actuator moved the two lick spouts in front of the mouse.

The fourth stage was the decision to lick the left or right spout. Animals were conditioned to lick right or left based on the type of tactile cue presented. In the case of aperture width, mice were trained to lick right on experiencing the wide aperture (2 cm), and left on experiencing the narrow aperture (1 cm). In the case of texture discrimination, mice were trained to lick right on experiencing rough texture and left on experiencing smooth texture

(Supplementary Video 4). After licking the spout, and obtaining a reward, mice moved backward in the lane, arriving at the center of the maze where a new trial could begin.

2.2.5. Bias correction

Mice performing 2AFC discrimination task typically develop a bias where they prefer one spout location over the other. To eliminate such a bias, our behavioral control software switched to a forced-mode whenever mice licked the same spout for 5 consecutive trials (Guo et al. 2014). In this forced-mode, the software dictated particular lane selection: only lanes with tactile cues related to the animals non-preferred spout were selected. The mouse stayed in the forced mode till it licked the correct spout for 3 consecutive trials. Once the animal showed that it correctly licked the non-preferred port for 3 trials in a row, the forced mode was terminated and the animal was switched back to randomized uniform selection of lanes.

2.2.6. Setup electronics and software design:

The setup consisted of **(Figure 13)**: 1) An Arduino-Uno microcontroller (www.arduino.cc) controlled and collected data from the Air-Track system; 2) A Pixy (CMUcam5) camera designed by Carnegie Mellon University and Charmed labs (Charmed labs, Austin, Texas) was used to track the platform location and orientation; 3) A 50 mm linear actuator with position feedback (Model L16 50N 35:1 12V Firgelli Technologies Inc., Canada) was used to advance the lick spouts to the mouse; 4) A capacitive touch sensor module (MPR121 Freescale Semiconductors Inc., Texas) was used to detect licking; 5) An active buzzer module (KY-012, KEYES DIY, China) was used for false alarm cues, in the case of incorrect licks; 6) Two solenoid pinch valves were used to release sugar water from reward tubes (2-way NC pinch valves, Bio-Chem Fluidics Inc., USA); 7) Data from the Arduino were sent via a serial connection to a python application running on a PC for logging and analysis.

2.2.7. Arduino code configurations

The Arduino code consist of the following elements **(Figure 14)**: 1) Each of the four lanes of the maze was defined by the angular orientation as determined from the angle of the rectangular red and green color label glued underneath the animal platform; 2) The beginning and the end of lane were defined by two coordinates that were fixed and used to determine whether the head of mouse was entering the lane or had reached the end of the lane; 3) Based on associating particular orientation co-ordinates for the platform with particular capacitive sensor and reward solenoid, the right and left lick spouts were defined in the code to assign a reward spout for each lane.

2.2.8. Behavioral measures from Arduino output and data analysis

An Arduino microcontroller polled inputs from the Pixy camera and licking sensors, while controlling solenoid valves, reward actuator, LED, and piezo buzzer. The solenoid opening time was set to 150 ms, which generated 10 µl reward. Decision time window was set to 3 s from the time the reward actuator was maximally extended until its retraction if no lick event occurred. The maximum travel distance of the motor was 5 cm (adding flexibility to the hardware design). The maximum travel distance used in the experiment was 3 cm and took 1.5 s on average. Reward access time: The time from the onset of licking the correct sensor till the retraction of the lick spouts was 2.5 s. False-alarm cue: A buzzing sound was delivered for 1 s after the mouse licked the wrong spout. Using values collected from the Pixy camera about platform movement, we set a fixed value in our code to report the status of the animal's location relative to the platform (i.e. lane range, lane boundary and end of the lane). Data was collected about the mouse's location, lane choice and licking decision from the Arduino serial port. The following definitions were used:

- A trial: Mouse started a trial when exited a lane (crossing a lane boundary) and ended when the mouse licked a spout or withdrew from the lane.

- Inter-trial interval: Time between the end of one trial when the mouse reported a decision by licking a spout and the time to move out of the lane, i.e crossing the lane boundary.

- Correct visual trial: Mouse chose to enter the dark lane (LED off). This counted as a correct visual trial whether or not the animal performed somatosensory task.

- False visual trial: Mice chose to enter the lit lane (LED on), even if it withdrew instantly afterwards.

- Correct somatosensory trial: Mouse in correct lane (LED off), licked the correct (rewarding) spout determined by lane aperture width or texture.

- Wrong somatosensory trial: Mouse in correct lane (LED off), licked the wrong (non-rewarding) spout.

- Miss somatosensory trial: Mouse in correct lane (LED off), did not lick inside the decision time window (3 s).

- Training session termination: When animals exhibited signs of satiation, stopped performing or exhibited a sharp decrease in behavioral performance over several trials (excluded from data analysis).

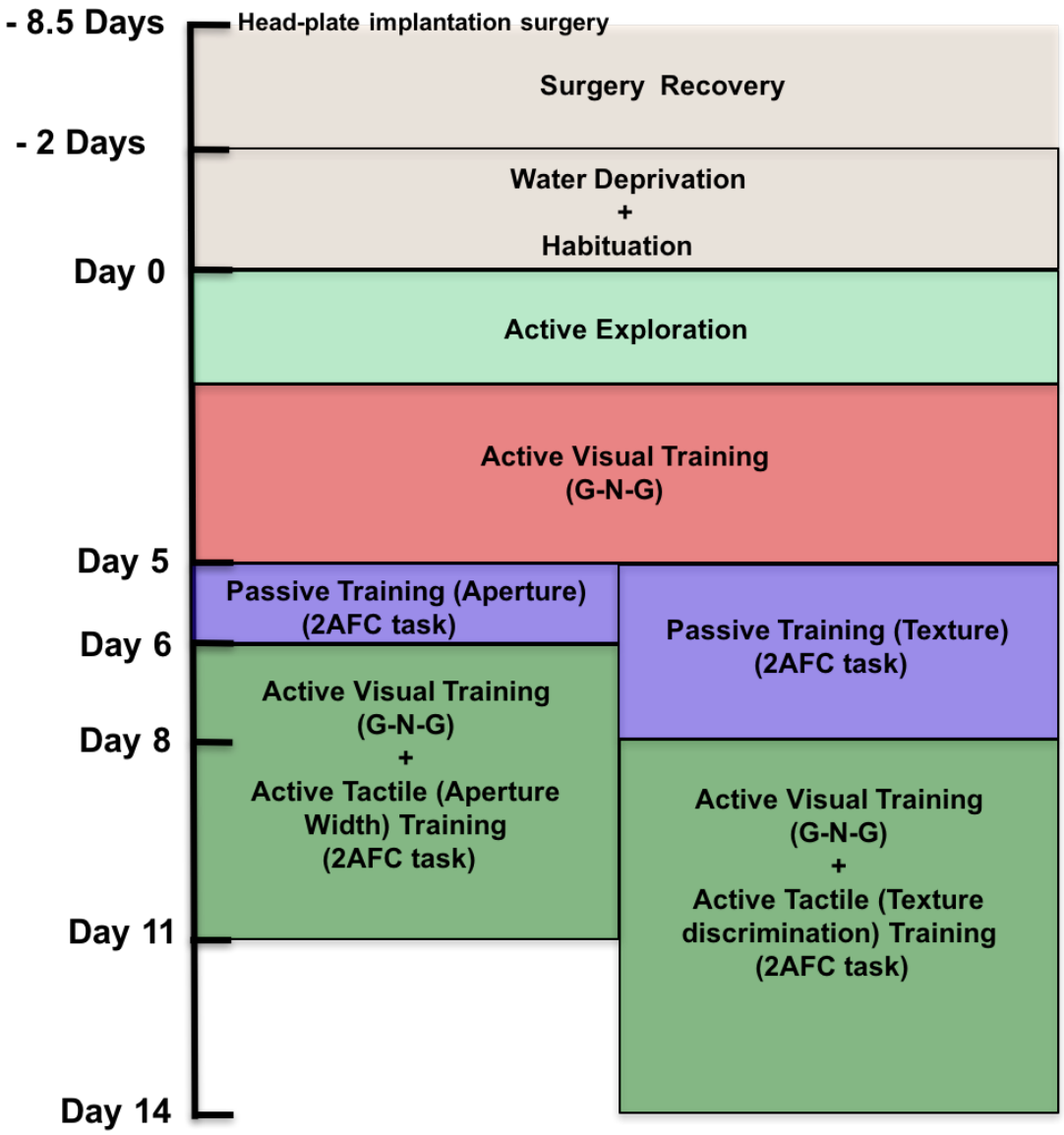

Figure 15. Training paradigm.

Duration of experimental phases; head-fixation, habituation and behavioral training along 2 weeks. During active exploration (pale green, sessions 1-2) the experimenter supervised the mouse while moving the plus maze and collecting rewards at each lane. During active visual training (red, sessions 3-4) 6 mice were trained to discriminate a visual cue until performance reached > 70%. During passive tactile training (violet, 1-3 sessions) mice were trained to navigate dark lanes and obtain rewards from a lick spout. 3 mice were trained to obtain reward corresponding to lane aperture width (1 session) in a two-choice task, while the other 3 mice obtain reward based on lane texture (3 sessions) in a delayed two-choice task. Finally, training advanced to combining active visual and active tactile training (dark green, 4-6 days) with mice trained to sequence of visual cue discrimination followed by tactile discrimination for either an aperture width or wall texture in the timeline of one trial.

2.3. Results

We designed and built an Air-Track system with a flat platform that floated on air, equivalently to most air-ball systems (**Figure 12**; see Methods). A physical maze was 3D printed on a platform (**Figures 12A, B**). The position of the floating platform was tracked with a video tracking system (**Figure 12C**;) and logged via an Arduino-Uno microprocessor (**Figure 13**) that also controlled the reward and cues in a closed loop system (**Figure 14**). It took about 2 weeks to complete the entire training paradigm, training individual mice to reach greater than 70% correct performance in visual Go/No-Go task and 70% correct performance in a two choice tactile task (either aperture width or texture discrimination task) (**Figure 15**).

For the purpose of testing the system, we chose a 'plus' maze with 4 lanes. We tested whether mice could navigate the maze, and perform visual and tactile discrimination in Go/No-Go and 2AFC tasks. In this environment, mice were trained to choose dark lanes (LED off) and to avoid lit lanes (LED on). Once mice entered a lane, their whiskers invariably touched the walls, following which the mice preceded to the end of the lane till they got a reward. Mice learned to discriminate between different aperture widths (marked green in **Figure 16A**) or wall textures, and decided between one of the two lick spouts (**Figure 16**).

To minimize stress for the animal, the task was self-initiated. Trials began when the mouse entered the center of the platform. **Figure 16** shows typical sensory-motor behavior on the "Air-Track' platform. Data from a single "typical" animal shows that the mean duration of the inter-trial interval from over 1000 trials for one animal was 11.5 seconds (**Figure 16C**). Mice typically spent equal amounts of time in the different arms, thus the data indicate that mice have no preference for any specific arm. (**Figure 16D**). This presumably reflected the symmetrical design of the plus maze. The average time spent in each lane was ~2.2 seconds (**Figure 16D**). During 1000 trials with 250 trials in each lane, this mouse spent 2.2 ± 0.6 s (mean ± SD) in lane one, 2.3 ± 1.2 s in lane two, 2.2 ± 0.5 s in lane three, and 2.1 ± 0.7 s in lane four.

As a mouse rotated the maze, the different lanes moved past it **(Supplementary Videos 1-4)**. In each trial the animal could move a single lane or multiple lanes, past it. Each lane it rotated past itself needed extra time (**Figure 16E**). Note that while there were only 4 lanes to choose from, animals sometimes missed the correct lane, which added time to an individual trial. This time interval varied considerably because of the duration of the various behavioral events within a trial – the time to pick a correct lane, the time to make a correct choice to lick right or left –could all vary (**Figure 16F,** mean and SEM for data from three animals). The mean durations for 3 mice sorted for the number of lanes they rotated past (only 4 lanes are shown in **Figure 16F**) show that many temporal aspects of the behavior -- for example the time taken to enter the correct lane, the time spent traversing a lane, and the time to make tactile decision and lick the spout -- were similar irrespective of whether the animal traversed 1 or 4 lanes before picking a lane. However, the duration of inter-trial intervals showed an inverse relation with the time the mice spent traversing lanes.

Having established that the animals could orient the Air-Track maze in a stress-free manner we extended the study to introduce two different kinds of behavioral tasks that could in principle be used to examine the correlation between neuronal activity and behavior. We did not train animals to the highest levels of performance, and have not yet measured from brain activity simultaneously, but within two weeks of consecutive days of training, animals reached threshold behavioral criteria in remarkably few sessions and trials compared to other typical head-fixed systems.

Six head-fixed mice were trained to perform in a Go/No-Go visual discrimination task, and achieved a criterion above 70% correct choices. Three of them were subsequently advanced to a second phase of training where they performed two choice aperture width discrimination tasks, while the other three were advanced to perform a delayed two choice texture discrimination task. The criterion for successful performance in this task was also 70%.

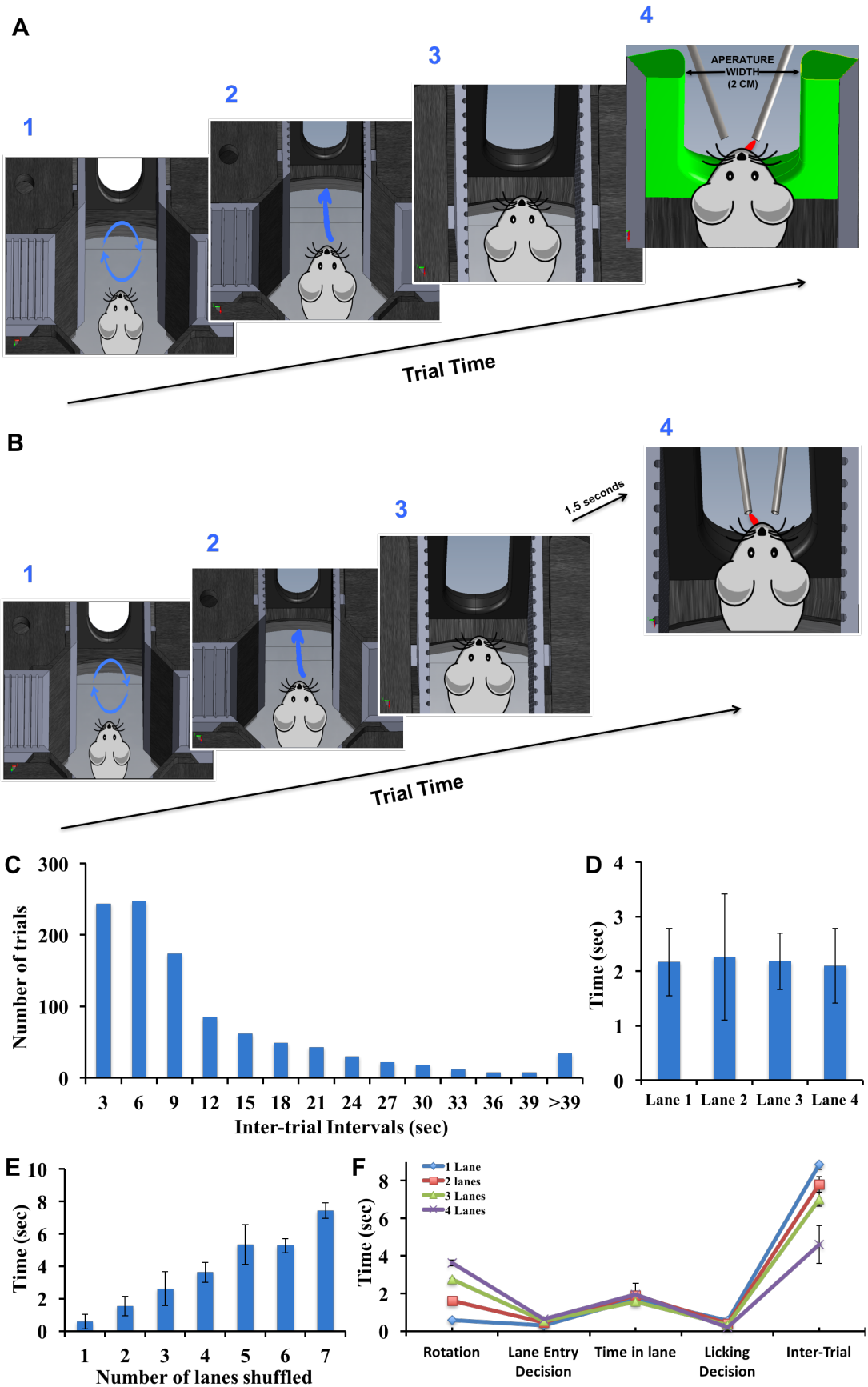

Figure 16. Task design and metrics in the course of the visual and somatosensory tasks.

Figure 16. A and **B** Schematic of task design for aperture width and wall texture discrimination tasks respectively showing that the mouse first rotates the plus maze (1), and watches the LED light cue which turns off when the mouse reaches the correct lane, and enters it (3). The final step is the discrimination of the aperture (marked green in the figure) or textures, with the decision reported at the end of the lane where the mouse licks one of the two spouts (4). In the case of texture discrimination, 1.5 second delay is imposed between the time the mouse reaches the end of the lane, to lick a spout. **C** Histogram of inter-trial intervals in 6 sessions of active training for a single mouse. Since the task was designed to be self-initiated, there was no fixed inter-trial interval. Mice could wait up to 30 seconds or more before initiating a new trial. **D** Average time spent by a single mouse in each lane from entering the lane to making a decision. The mouse spent ~ 2 seconds in each of four lanes before obtaining reward. **E** Time spent by a single mouse rotating to reach the correct lane. Time spent rotating increased with increasing the number of travelled lanes. Note that mice can traverse clockwise or counterclockwise, and as they traversed additional lanes, they could take more than 7 seconds finding the correct lane. **F** Average durations of behavioral events for 3 mice. Trials are sorted according to the number of lanes mice rotated around themselves (blue, one lane (n = 1550 trials); red, two lanes (n = 361 trials); green, three lanes (n = 350 trials); and purple four lanes (n = 20 trials). The time points picked for these analysis are 1) rotation time, which increased with the number of lanes mice rotated; 2) visual reaction time to enter a correct lane which started when the light turned off and ended when the mouse crosses the lane boundary; 3) time spent inside the lane which began when the mouse entered the lane and ended when the mouse licked the reward spout; 4) tactile reaction time to lick the right or left spout; 5) inter-trial interval between trials determined by the motivation of the mouse. When mice spent more time rotating past additional lanes, they spent less time before starting a new trial.

The goal of rotation was to find and choose the correct lane, which was selected on the basis of a visual stimulus. After three days of training mice (n=6) learned to use this visual cue and select lane where an LED turned off with a mean performance of 84.5% correct choices (**Figure 17A**). **Figure 17B** shows performance over 4 days and for up to 1000 trials for 3 mice. During the initial phase of training (day 1), animals were guided (supervised) manually to choose the correct lane (**Figure 17B,** manual guidance is shaded in grey color).

The aim of the supervised phase was to maintain the animals' motivation to perform more trials per session in this early phase of training. Mice reached 70% correct performance within 300-700 trials (**Figure 17B**).

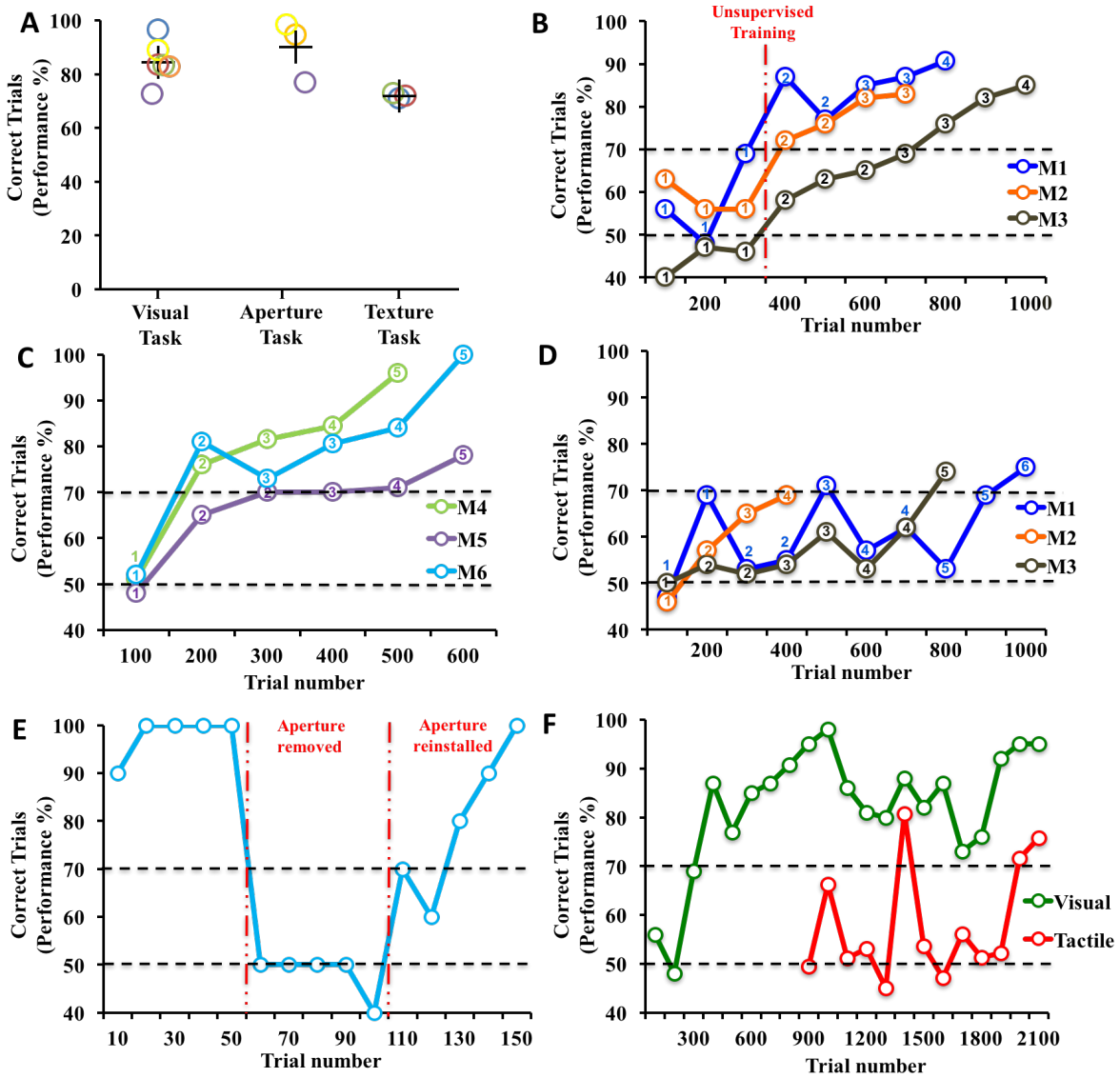

Figure 17. Mice performance in visual and tactile discrimination tasks in Air-Track.

A Performance in a Go/No-Go visual discrimination task and in 2AFC tactile discrimination tasks. The average performance in the visual task (n = 6 mice) was 84.5% correct trials after 4 days of active visual training. The average performance in the aperture width tactile task was 91% (n= 3 mice), while average performance was 72.6% in texture tactile task (n= 3 mice) after 4-6 days of active tactile training. **B** Performance of 3 mice in the Go/No-Go visual discrimination task within 4 days of training. Mouse 1 (M1, blue line) achieved 91% rate; Mouse 2 (M2, orange line) achieved 83%, and Mouse 3, (M3, brown line) achieved 85%. During the 1st day of training, the experimenter provided fine control guiding the animal to keep mice motivated (shaded in grey); afterwards the animals were unsupervised.

C Performance of 3 mice during the two-choice aperture width discrimination task. In 5 days, mouse 4 (M4, pale green line) achieved 96% success rate, while mouse 5 (M5, Violet line) achieved 78%, and Mouse 6, (M6, skyblue line) achieved 100%. **D** Performance of 3 mice during the delayed two-choice texture discrimination task. Mouse 1 (M1, blue line) achieved 75% success rate in day 6; Mouse 2 (M2, orange line) achieved 69% in day 4, and Mouse 3, (M3, brown line) achieved 74% in day 5. **E** Mouse performance during active aperture width tactile discrimination through the timeline of one session. Tactile performance reaches 100% (data shown for M6) before it drops to chance level after removing the difference in aperture width between lanes. Reinstalling aperture width differences between lanes retrieves correct behavioral performance. **F** Cross-modal performance of visual and tactile tasks during multiple session of training. Visual discrimination performance declined (data shown for M1) from 90% to 75% as it learned the texture discrimination task. Over several days, performance increased above 90% success rate in the visual discrimination task, while performance in the texture discrimination task reached 75% on day 6.

Next, mice were advanced to perform a more difficult task that combined the visual task Go/No-Go with one of the tactile 2AFC tasks (**Figure 17A**). During the aperture 2AFC task, the three mice reached the 70% correct choices by the second day (200-300 trials) and maintained this accuracy for the next three days (**Figure 17A, C**). In the texture delayed 2AFC, two mice reached 70% correct choices within 4 to 6 days of training (400-1000 trials) (**Figure 17A, D**). To prove that mice use the aperture apparatus in the two-choice task, we measured the mouse performance (data shown for M6) with and without the aperture apparatus during the timeline of one session (**Figure 17E**). Mouse tactile performance reaches high level before it drops to chance level after removing the aperture apparatus from all lanes. Reinstalling aperture apparatus retrieves correct behavioral performance. (**Figure 17F)** shows cross-modal performance (data shown for M1) along different training sessions performing visual and tactile discrimination tasks. The visual performance declined initially after the introduction of the tactile discrimination task before visual and tactile performance increase proportionally at the end of the training sessions.

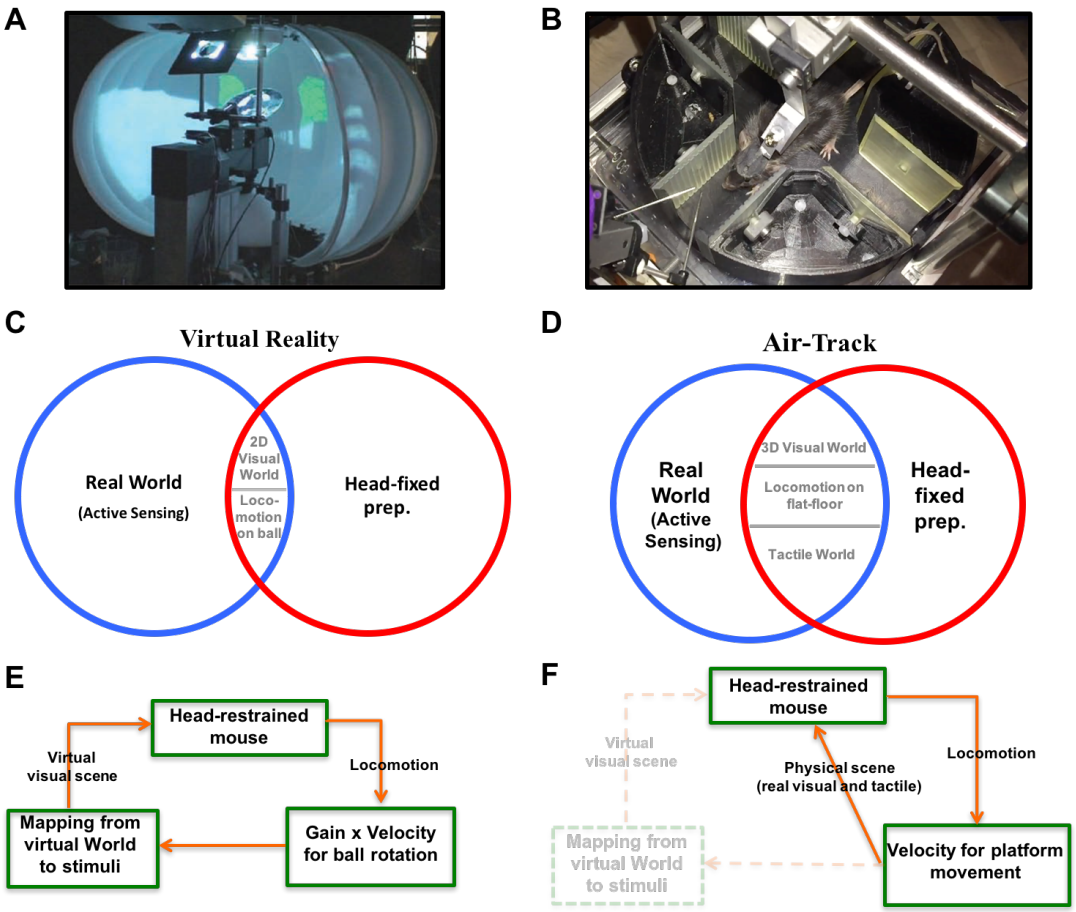

Figure 18. Air-Track provides a real-world experience in head-restrained rodents.

Virtual reality platform adapted from Dombeck and colleagues (Dombeck et al. 2009) **A** and an Air-Track platform **B**. Air-Track provides a real maze, while virtual reality creates a virtual maze. In both systems the walls and the shape of the track can be changed based on the experimental design. **C** and **D** key difference between an Air-Track and a virtual reality setup. Air-Track offers more of the real world to head-fixed mice, as it contains a palpable world with multiple dimensions; the air balls and virtual realities typically generate a visually rich but non-tactile world. **E** and **F** Experimental mapping with a virtual reality and Air-Track. Air-Track can create a real environment with walls that can be used to skip the mapping of sensory input and motor output. In addition, a virtual visual world can still be introduced to surround the Air-Track as with air-floating track spheres.

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2.4. Discussion

We present a novel behavioral system for use with head-fixed mice. The Air-Track is flexible, low-cost, easy to build, and requires only a minimal computational control and data acquisition system when compared to common virtual reality systems. It is unique in providing a behaviorally rich environment for active sensing that engages multiple sensory modalities simultaneously. The proof-of-principle testing of mice in this environment provided by this study shows that this system can be used to quickly train animals for both simple and complex tasks.

Air-Track exploits the principle of using air-lifted platforms previously described by Kislin and colleagues (Kislin et al. 2014). Several features of the Air-Track system are novel, introducing major engineering and design elements that make it amenable for behavioral automation in active sensing experiments. First, we used a camera-controlled real-time tracking of 2D position and rotation of the floating platform. Second, we developed an automated closed-loop hardware/software control system based on an Arduino interface that provides an interactive environment to control stimulus presentation and reward delivery. Third, we designed a versatile reward delivery system appropriate for different physical environment configurations. Fourth, we utilized 3D printing technology to construct mazes for flexible design of novel environments. Using our automated closed loop system we demonstrated that animals could be easily trained on novel behavioral tasks with multimodal stimuli (visual, somatosensory and auditory).

The Air-Track system is most directly comparable to virtual reality systems that also attempt to achieve quasi-natural behavior in rodents while head fixing the animal on an air-cushioned ball (Harvey et al. 2009) (**Figure 18A, B**). Virtual reality approaches have been extremely successful in revealing precise information about brain activity during active sensing tasks. Most studies to date have focused on visual-motor tasks, typically running through a visually displayed virtual-maze (Kaneko and Stryker 2014; Keller et al. 2012; Saleem et al. 2013). For instance, such systems have been used to show how ensembles of neurons are recruited in the visual system while

learning new environments (Poort et al. 2015) and how different behavioral states such as arousal influence visual processing and functional flexibility of V1 (Vinck et al. 2015). An alternative approach applied to the vibrissal sensory motor system has been to mount a tactile environment – a set of walls -- that moves as the animal walks on an air ball (Sofroniew et al. 2014). Generating these kinds of complex environments is difficult to achieve in a traditional head-fixed, non-active sensing approach.

In our behavioral design, we used three sensory discrimination tasks; visual Go/No-Go task, 2AFC aperture-width discrimination task, and a delayed 2AFC texture discrimination task. In visual and aperture discrimination tasks, the mice learned the task in few days, while in the texture discrimination task mice performance was uneven. A major cognitive challenge in performing the texture discrimination task was that it also had a working memory component. After contact with the stimulus, i.e. wall texture, the mouse had to extend its head outside the lane, losing contact with stimulus for 1.5 s till the decision could be made. This task therefore required the retention of the texture in working memory, which significantly increased the difficulty of the task. Although the mice passed the 70% criteria in the delayed 2AFC texture discrimination task in a few days, their performance across sessions was worse and uneven compared to the other two tasks.

The Air-Track provides a convenient “one-size-fits-all” solution that extends virtual reality approaches to ultra-realistic and multi-modal behavioral regimes. Whereas virtual reality approaches are best suited for visual stimulation, the Air-Track system automatically includes multiple modalities (**Figure 18C, D**). That is, since the maze is physically present, all of the possible sensory information usually available to freely moving animals (visual, auditory, tactile and olfactory) is available. We did not explicitly explore all modalities in this study (concentrating on vision, and somatosensation), however animals also had auditory (buzzer) (and in principle could have had olfactory) cues coupled to their movements in the maze.

Another important difference between virtual reality approaches and the Air-Track is that virtual environments require the mapping of the animal's movements to the virtual world, which can only be done via a computer model (**Figure 18E**) involving an additional step in the sensorimotor loop. With the Air-Track, this step is unnecessary as movements of the mouse are automatically translated into movement of the physical maze (**Figure 18F**). This has several advantages: 1) there is an accurate coupling of the animal's movement with the movement of the environment with no 'glitches' (i.e. computational mapping errors), 2) there is little subjectivity in determining this mapping and 3) it requires zero computational effort or expensive equipment. On the other hand, a disadvantage of our approach is that deliberate mismatches in mapping are more difficult to produce as compared to what can be done in virtual reality systems that take the animal's internal model (mapping) of the virtual world into account (Keller et al. 2012; Saleem et al. 2013). It may still be possible with Air-Track to introduce pseudo-mapping errors (i.e. the animals internal model) by changing the friction (air pressure) or disturbing the movement of the platform.

Another disadvantage of the Air-Track system is that the mazes are fixed and finite compared to virtual mazes that can be infinite and/or freely changing. We used a 15 cm diameter platform, which provided enough space for several lanes, and this this may compromise the effectiveness of the Air-Track system for experiments exploring the activity of place cells. However, larger platforms that mice can move are likely to engage activity of place cells, and are likely to engage an animal's sense of place. Consequently, it should be possible to use the Air-Track in multi-modal spatial tasks (Gener et al. 2013; Griffin et al. 2012).

A practical limitation of the Air-Track is that the available space and the inertial load of the platform for the animal, limit the maximum dimensions of the system. The inertial load in our current design was extremely low (**Supplementary Video 1**) such that movement appeared completely normal. In fact, we found it necessary to introduce some artificial friction by reducing

air pressure below maximum to better approximate the animal's inertia in a natural, freely moving situation.

The movement of the animals in the Air-Track system is likely to be more realistic than on a ball or treadmill because the platform is flat. In addition, the flat surface of the platform can be used for different kinds of somatosensory cues, textures on the floor of the maze, or directional auditory cues for a Y-maze that have proved convenient in freely moving mazes (Manita et al. 2015). Furthermore, with Air-Track we can deliver a rich, complete environment with different textures or aperture widths that previously were typically used in freely moving animals (Chen et al. 2015; Jadhav and Feldman 2010; Krupa et al. 2004; Prigg et al. 2002; von Heimendahl et al. 2007; Wolfe et al. 2008). But just as with most head fixed preparations, it is necessary to keep the Air-Track system perfectly horizontal, which prevents some possible experiments, in particular tests of vestibular contribution, however, this is an intrinsic difficulty for all head-fixed systems.

The tracking system we chose was the "Pixy" camera attached to an Arduino-Uno microprocessor which provided convenient off-the-shelf tracking of the coordinates of the platform in real time. In principle, more sophisticated and high speed tracking systems could also be used. We placed an emphasis in our study on easy-to-obtain components with the intention of making the system easily available to any laboratory. For this reason, the code is open source and hardware/software descriptions are available online at "http://www.neuro-airtrack.com". Since the system is small and compact, it can be introduced into practically any existing recording setup (e.g. under most *in vitro* microscope systems) and therefore can easily be moved from one setup to another within a laboratory to maximize the recording strategies. The total material cost of our entire system was in the range of €200-€500 (~US$300-$600) depending on the materials used and manufacturing costs (e.g. 3D printing). We made several mazes including the plus-maze presented here in this paper and a y-maze. Other shapes could be easily and flexibly used using common 3D printers.

Given the simplicity of the system and its open source availability, there should no barrier against its introduction into any neuroscience laboratory interested in behavioral paradigms based on active sensing.

Chapter 3: Pixying behavior: a real-time and post-hoc automated optical tracking method.

3.1. Introduction

A traditional approach to the study of neural function is to relate activity in a circuit to a distinct behavior. While methods for measuring and manipulating neural activity have become increasingly sophisticated, the ability to monitor and manipulate behavior in real-time has not kept pace. Even today, even in some of the most sophisticated closed-loop behavioral electrophysiology and imaging systems i.e. visual virtual reality where motion of the treadmill or air-ball is used to remap the visual world, there is no direct report of the animal movement; the motion tracked is that of the treadmill or the air ball (Dombeck et al. 2007; Harvey et al. 2009).

To overcome these kinds of limitations in behavioral monitoring we used the whisker system, a system that offers many advantages for understanding sensory systems and has been responsible for key advances in monitoring neural activity i.e. calcium imaging of neurons and dendrites in vivo, imaging activity of axons, whole cell patching in behaving animals etc. (Gentet et al. 2010; Lee et al. 2006; Petreanu et al. 2012; Svoboda et al. 1997; Svoboda et al. 1999). In these types of experiments, a variety of approaches are used for monitoring whisker movement during behavior (2013; Hentschke et al. 2006; Sofroniew and Svoboda 2015; Zuo et al. 2011). High-speed videography is one common approach (Arkley et al. 2014; Carvell and Simons 1990; Clack et al. 2012; Grant et al. 2009; Hartmann et al. 2003; Knutsen et al. 2005; Ritt et al. 2008; Sachdev et al. 2001; Voigts et al. 2015; Voigts et al. 2008). Another approach is to use electromyography (Berg and Kleinfeld 2003a; Carvell and Simons 1990; Fee et al. 1997; Sachdev et al. 2003; Zagha et al. 2013). Alternatively, an array of sensors or a single laser / IR sensor has been used for tracking the movement or position of a whisker (Bermejo et al. 1996; O'Connor et al. 2013). Each of these approaches has

advantages and disadvantages. High-speed imaging has unmatched spatial-temporal resolution; it can be used for monitoring one or multiple whiskers at a time, but it is typically not used in real-time or in feedback mode. In addition, it's inflexible as most tracking algorithm are customized to track a distinct object in a very specific setting that is hard to replicate by different laboratories. Furthermore, high-speed imaging usually requires an expensive camera and involves enormous amounts of data that are time-consuming and cumbersome to analyze post-hoc. EMG of the facial pad muscles can provide real-time feedback about whether the animal is whisking, but it is invasive and does not provide sufficient spatial resolution for monitoring the location, set point or angle of individual whiskers. Beam breaking / CCD array methods have high temporal resolution but are inflexible and typically designed for monitoring the location and motion of a single whisker.

In this study, we present a method that turns an off-the-shelf camera (helped along by customized software) into a real-time optical tracking system for use in any behavioral electrophysiology setting. We can quantify the distance moved, the speed of movement, and the location of the body part or of the whole animal. The Pixy camera package can also be combined with high-speed cameras, forming an offline tracking system with high temporal resolution limited only by the high-speed camera recording frequency. The easy integration of these two systems increases the flexibility of tracking; it reduces the costs, and makes it possible to analyze large quantities of video data.

3.2. Methods

3.2.1. Surgeries

All animal procedures were performed in accordance with the animal care committee's regulations. Mice were maintained in a reverse day night cycle environment throughout the course of the experiments. Eight adult mice were surgically prepared for head restraint by attaching a head-post to the skull under Ketamine/Xylazine anesthesia (90 mg/10 mg/Kg). In the two days after surgery, Buprenex analgesia (0.1 mg/Kg) was administered and the animal health was monitored. Rely-X cement was used to affix the head-post to the skull (Applicaps, 3 Com, USA) (Andermann et al. 2013). In two animals, a lightweight detachable Styrofoam color ID was affixed to the head-post to enable tracking of the freely moving animal.

3.2.2. Animal training

One to two weeks after surgery, animals were habituated to head fixation on a stationary platform, or to head-fixation on a treadmill or were allowed to explore a clear linear 42 cm long x 9 cm wide track made of Styrofoam. In subsequent days, animals were head-restrained for short periods of time, while individual whiskers were painted by dabbing UV sensitive body paint (UV Glow, Germany) mixed with super glue. Mice were habituated to the coloring of whiskers and the placement of a piezo-film sensor at some fixed distance from the whiskers (Bermejo and Zeigler 2000; Sachdev et al. 2001). Whisker contact with the sensor was rewarded with a drop of sweetened condensed milk. Mice were trained to move their whiskers in response to a sound cue (**Figure 19**). Whisker contact of sufficient force against the piezo-film sensor elicited a reward (**Figure 19B**).

In the second task, animals were habituated to head-fixation while on a treadmill. The forepaws were painted with two different UV dyes one for each paw. For freely moving animals, a piece of multi-colored Styrofoam (different colors combination for each animals) was glued to head-post and used for tracking mice in regular light conditions. In all paradigms, animals were water

restricted and weights were monitored daily and maintained at >85% body weight.

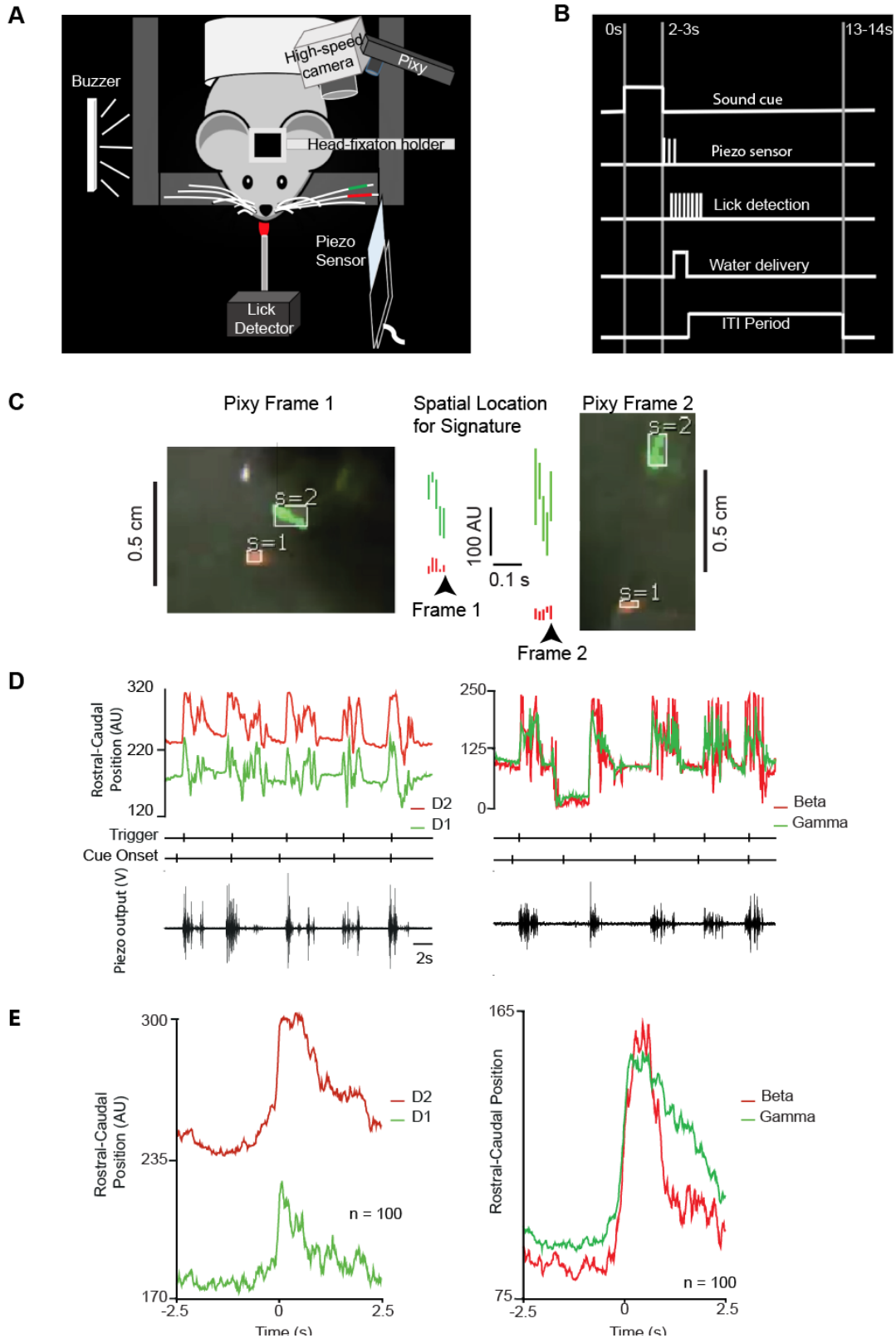

Figure 19. Pixy-Tracking system for whisker tracking.

Figure 19. A Setup design. Head-fixed mice are acclimatized to whisker painting, and trained to use their whiskers to contact a piezo-film touch sensor. A Pixy camera is used to track whiskers in real-time (left), a high-speed color camera is used simultaneously to acquire data. **B** Paradigm for whisker task. A sound-cue initiates the trial. The animal whisks one of the two painted whiskers into contact with a piezo-film sensor and if contact reaches threshold, the animal obtains a liquid reward. There is a minimum inter-trial interval of 10 seconds. **C** Capturing whisker motion in real-time. The movement and location of the D1 and D2 whiskers shown at two consecutive time points (20 ms apart, left & right images). Lines corresponding to the location of the two whiskers (middle panel) acquired with Spike2 software. The waveform of whisker data reflects the spatial location and the dimensions of the tracked box around the whisker, which can both change as the whisker moves. **D** Real-time Pixy data from D1 and D2 whiskers (left, raw and smoothed) or Beta and Gamma whiskers (right, smoothed), as a mouse performs five auditory go-cue triggered trials. Mice move a whisker into contact with a piezo-film sensor (bottom). Contact with the sensor triggers a reward. The cue onset and the reward trigger times are marked below the whiskers movement traces. Note that the spatial location of the D1 and D2 whiskers is distinct; the position of the two whiskers rarely overlap. In these trials, the distance between the two whiskers ranged from ~ 2-10 mm (distances converted into arbitrary units that denote spatial location). **E** Average position during task performance. The D1 and D2 whiskers move differently (left): the average position of the two whiskers at rest is different (before zero), and the average position of the two whiskers at contact is different (at zero). The D2 whisker, which contacts the piezo-film sensor and is rostral to the D1 whisker, moves more than the D1 whisker. In contrast, the two arc whiskers' position overlaps at rest and at contact.

3.2.3. Experimental setting

A Pixy Camera (Charmed labs, Carnegie Mellon University) was equipped with a 10-30 mm f1.6 IR lens and connected to the USB port of a computer. Pixy uses an HSV (hue, saturation, and value) color-based filtering algorithm to track colored objects. The open-source camera software, Pixymon, was used to mark up the colored whiskers and limbs defining a distinct signature for each color. Color signatures were tuned to achieve consistent tracking without generating false positives (detecting wrong objects) or false negatives (detecting the object intermittently or sparsely). Pixymon software enables signature tuning via its configure dialog tab, where signatures' tolerance can be optimized by adjusting a set of graphical sliders. The camera can learn up to 7 color signatures and can be set up to distinguish multiple objects of the same color signature.

We modified the open-source Pixymon software to send a set of text values over a user datagram protocol (UDP) network port. The text values report the x, y coordinates of the tracked object. From day to day, the coordinates (units) can vary because of positioning of the camera, the precise zoom used on the camera, and the angle of the camera. In the case of the beta gamma whiskers, which are arc whiskers, there is considerable overlap in position of the whiskers relative to the camera **(Figure 19)**. The data can be imported into any data acquisition software. Here we use Spike2 (CED, Cambridge) for data acquisition. A Spike2 script is used to transform the *x* and *y* text coordinates into waveforms. The modified Pixymon software, the spike2 scripts as well as further scripts are available online: www.neuro-airtrack.com/pixy_paper/pixy.html

3.2.4. Data acquisition

Painted whiskers or limbs or color ID on the animal head showed continuous tracking without saturation or breakdown. Pixy adapts to a variety of light conditions, including dark-ultraviolet, infrared, incandescent (reddish hue), or fluorescent (bluish hue) light. The white balance for each lighting condition is automatically adjusted as the Pixy powers on. When light conditions change, the white balance can be reset by unplugging the Pixy camera or by pressing the reset button for 2 seconds. In dark light, we use no more than 3 colors. In IR light, a whisker was painted with fluorescent dye and tracked using illumination from an infrared light source (Thorlabs, Newton, NJ). On the treadmill, the same methodology was applied for tracking forepaws (one color for each paw). For freely moving animals, we tracked the head direction using multi-color signatures, called a “color code” with which object position and angle can be automatically tracked. For offline tracking, a Basler high-speed color camera (Model number acA1920-155) was used to capture images at 155 Hz. The high-speed camera recordings were played back in slow motion on a screen while the Pixy camera was setup to track the colored objects off the screen.

3.2.5. Data analysis

The real-time data from Pixy was mapped to Spike 2 channels. When combined with the timing of behavioral events it is possible to take single trial (touch triggered or go-cue triggered) data for two adjacent whiskers and to make average waveforms for all movement data for each whisker over multiple trials. To show that both the *x* and *y* coordinates could be monitored by Pixy we sampled the *x* and *y* coordinates of limb position and mapped this to Spike2 channels. In freely moving animals, the head rotation angle and *x* / *y* coordinates of animal position were acquired into spike 2 channels and converted into a linear track of movement of the animal, or into heat maps of the animal. For the heat maps, we constructed a 2 dimensional histogram of pixels in each video frame, and applied 100 rounds of spatial filtering, where each pixel's value was recomputed as the mean value of the pixel and each of its adjacent pixels (n=8). Finally, high-speed video acquired at 150 Hz was played back at 6 Hz, and Pixy was used to capture the movement of whiskers into a spike2 channel.

3.3. Results

We used the Pixy-based system on head-fixed mice (n=6). 5 mice had their whiskers painted with UV-fluorescent paint and 1 mouse had both forelimbs painted (see Methods). A high-speed camera and a Pixy camera were positioned to track two whiskers (**Figure 19A**)**.** In this paradigm, mice were conditioned to whisk in order to contact a piezo-film sensor after a go-cue turned on (**Figure 19B**). To ensure that the painted whiskers were used in the contact task, the large whiskers rostral to the painted ones were trimmed off. We first determined whether the real-time whisker motion captured in video frames matched the position data recorded in real-time (**Figure 19C**). Video synchronized to the real-time data provided by Pixy indicated that both the absolute (real) and relative (x, y coordinates in the Pixy frame) whisker positions were tracked accurately (**Figure 19C middle**). In frame 1, the two painted whiskers are close to each other, in frame 2 both tracked whiskers are further apart. The total movement (in 20 ms) of the two whiskers is reflected in the length of the lines (**Figure 19C, middle**) and the location of the red and green traces (lines) reflects the position of the whiskers in the two frames.

Next we used these methods to track two adjacent whiskers (**Figure 19D, Supplementary Video 5**). The D2 and D1 or the beta and gamma whiskers were tracked in the course of five cue-triggered contacts. The mouse used the D2 or the beta whisker to touch the piezo-film sensor. These five contact trials show that at rest and during contact with the piezo-film sensor, the position of D2 whisker rarely overlapped (<1 mm) with the D1 whisker (at least at the point where the two whiskers were painted). While the two whiskers position was distinct and non-overlapping, the motion of the whiskers was in phase with each other. In contrast, when the arc whiskers (beta and gamma) were tracked (**Figure 19D, right**), the whiskers showed considerable overlap in the rostro-caudal position. These data indicate that the spatial location of the whiskers can be accurately tracked. Next we generated whisker touch triggered averages of movement of the two painted whiskers in each animal (**Figure 19E**). These experiments show that the whisker that touched the sensor (D2 or beta) moved to a greater extent, i.e.

there is a larger deviation from rest on average for the whisker used to elicit touch-triggered reward.

The real-time temporal resolution of 50 Hz is borderline for the use of the Pixy camera for fast movements of the body, fast movements that include whisking, which in mice can reach 25 Hz. We therefore developed and validated another approach – an automated, offline, slow motion approach using an additional high-speed video camera that is often used to faithfully track whisker motion. The recorded high-speed video behavior was played back on a computer monitor in slow motion and a Pixy camera was positioned in front of the monitor to track the colored whiskers (**Figure 20A, Supplementary Video 6**). For a fraction of cue-triggered trials, we compared the Pixy camera tracked slow motion data to the simultaneously acquired real-time data (**Figure 20B**). Surprisingly, the real-time and the offline slow motion waveforms are qualitatively similar, the position of the two row whiskers does not overlap at rest or during contact, and the envelope and duration of movement of the adjacent whiskers looks similar in both conditions. This implies that for some purposes, the Pixy camera approach is appropriate. Nevertheless, the higher temporal resolution tracking of the offline video shows that the high frequency components of the movement are not captured in real-time by the Pixy camera.

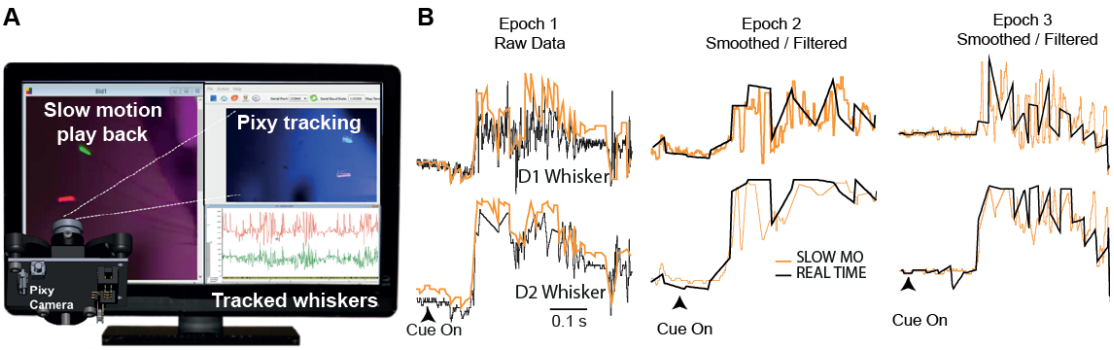

Figure 20. Pixy camera for post-hoc automated tracking.

A Diagram of a Pixy camera capturing whisker motion previously recorded with a high-speed video camera and played back in slow motion on a monitor. **B** Comparison of the high-fidelity signature recaptured automatically by the Pixy camera in slow motion (orange) with the data acquired in real-time (black).

To examine whether this method can be extended to infrared light condition (invisible to rodents), we painted a whisker with the same UV body paint, but instead of using UV dark light or regular illumination, we illuminated the whisker with infrared light. For proper IR illumination of just the whisker, the angle of the infrared light was key: the IR light was positioned under the Pixy camera, and directed at the mouse whisker pad from the side. A single, painted whisker was tracked using a Pixy camera (**Figure 21A, Supplementary Video 7**). Turning the infrared light off, removed all position information in the output. The text marks, and the Y position information were

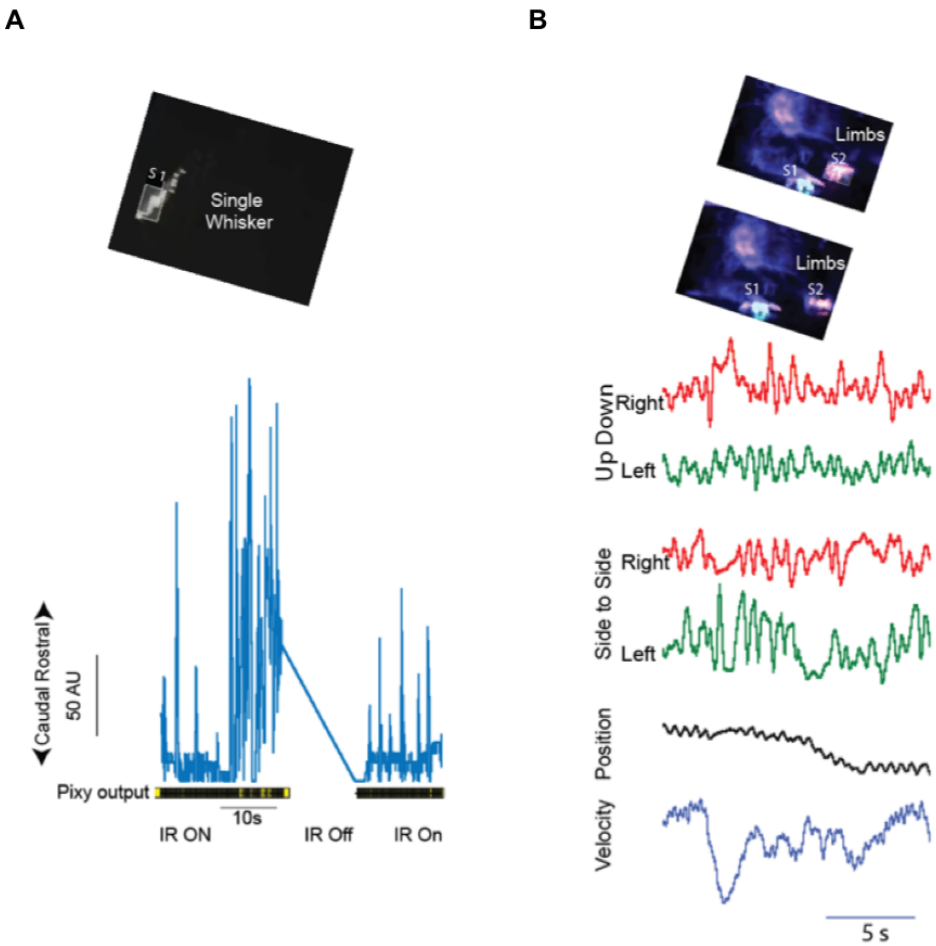

Figure 21. Pixy in infrared light and for tracking limb movement.

A Top, Pixy image of whisker painted with yellow UV light sensitive paint, illuminated with infrared light only and automatically tracked in real-time. Bottom, output from Pixy camera showing periods with (IR ON) and without (IR OFF) illumination. **B** Pixy tracking of two limbs. Two frames showing one limb painted green, the other painted red tracked with a Pixy camera. The output from the limbs show the motion in both the x and y coordinates i.e. up down and side to side. The position and velocity of the treadmill are also recorded in real-time.

no longer generated and were no longer evident as a waveform. When the IR light was turned back on the real-time whisker motion was reacquired and tracked without any additional adjustment.

To demonstrate the flexibility of the Pixy camera system, we used it to track both forepaws of mice on a treadmill. The paws were painted with different colors, and the Pixy camera was positioned at the height of the forepaw of a mouse (**Figure 21B, Supplementary Video 8**). In this configuration, we tracked each forepaw distinctly in both *x* and *y* coordinates simultaneously: i.e. the Up and Down and side to side motion of the paws was captured in real-time as the animal moved on the treadmill.

Finally, we used Pixy to track head rotation and *x* / *y* coordinates of freely moving animals position in a 42 cm x 9 cm wide box in real-time (**Figure 22A, B**, **Supplementary Video 9**). The moment-by-moment changes in head angle and animal location data (*x* and *y* coordinates) can be transformed into waveform (**Figure 22A**) where F1 (related to the vertical position of the animal in frame 1 on the right) is at the bottom and has a value close to zero. In frame 1, the animals head angle is horizontal, in frame 2 the angle rotates by ~70 degrees, in frames 3 and 4 the angle is rotated by 180 degrees (compared to frame 1, **Figure 22A**). The side to side position of the animal changes, with the animal sometimes hugging the right side (frames 1, 3), the left side (frame 2) or is roughly in the middle of the box. The position of the animal can be traced at 50 Hz (**Figure 22B**) and a heat map of the animal location in the box over 3 minutes of tracking can be constructed. In addition to tracking the location of individual animals, Pixy can be used to track multiple color IDs affixed to the animal head (**Figure 22C**), thus simply and flexibly tracking one or multiple distinct freely moving animals.

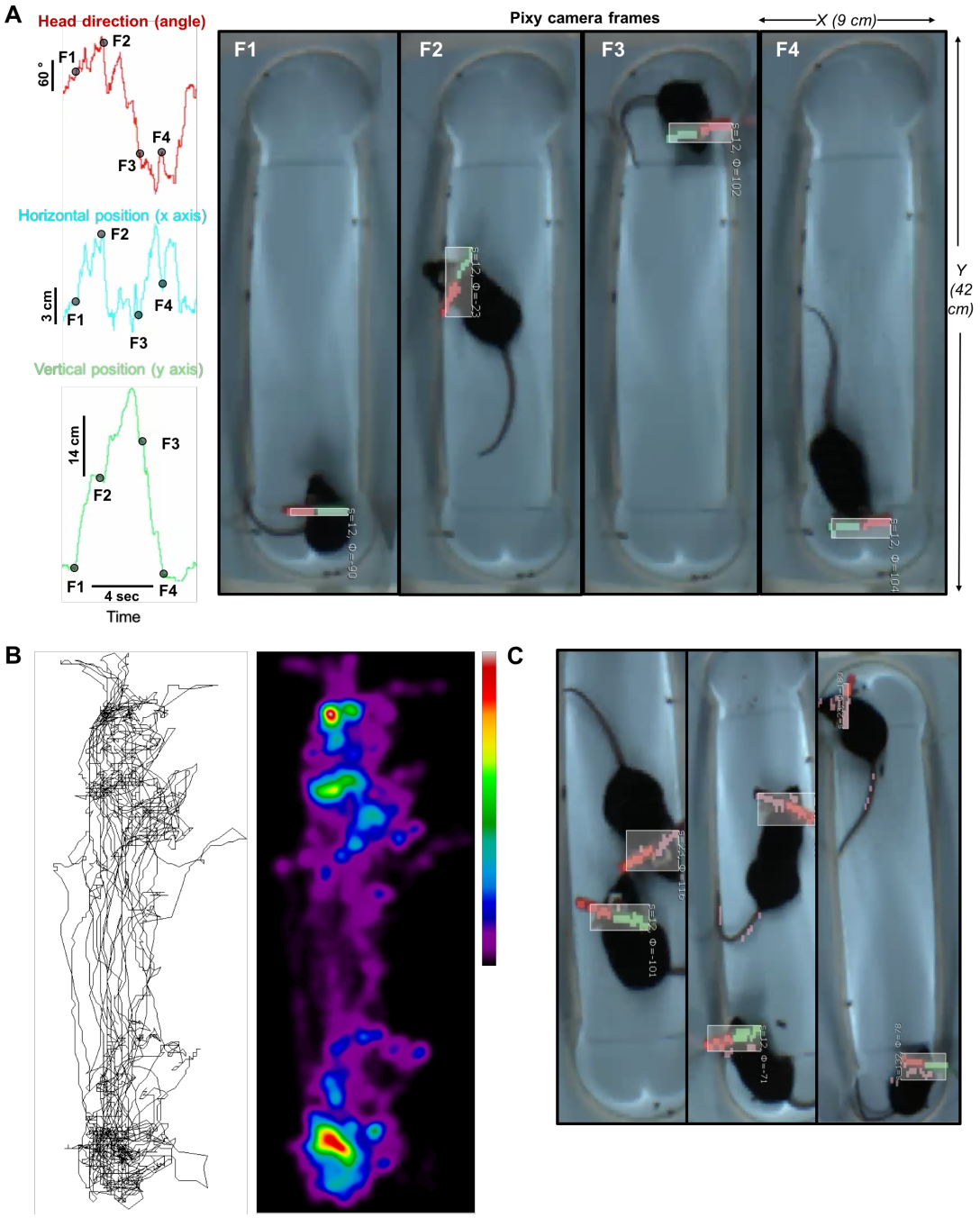

Figure 22. Tracking head rotation and location of freely moving animals.

A The head rotation (top), x (middle) and y (bottom) coordinates of animal position were simultaneously tracked. Four time points corresponding to the four frames are shown, where the animals head direction, and position in the box change from moment to moment. **B** The animal's position over 3 minutes was tracked and a heat map of the preferred location was created, red = more time, blue = less time. **C** The location of two animals in the same enclosure can be distinctly tracked, including each animal head rotation, and position. Pixy tracking is shown by the boxes around the animal's head.

In further experiment we decided to exploit the advantage of the Pixy-Tracking system for tracking whisker movement on the Air-Track system (**Figure 23**). Using the Air-Track system, mice stand of a moving platform with walls. With the movement under and around the animal, tracking whisker movment is a daunting task to combine Air-Track with other common tracking algorithm that track whisker geometrical pattern. The Pixy-Tracking system utilizes a color-filtering algorithm that makes track the whiskers on the Air-Track system plausible. Here, we are primarily interested in showing the power of combining both behavioral systems to quantitatively understanding complex behavior. Further studies will be performed to study whisker kinematics on the Air-Track system.

Our preliminary experiments (**Figure 21**) illustrate that it is possible to track tracking multiple whiskers (one whisker on each side of the animal) of a mouse navigating a plus-maze in the Air-Track. The colored whiskers are easy to distinguish from the background and their movement is informative about the entire sequence of behaviors monitored during the discrimination task. Here, we used high-speed camera to collect frames of mouse movement and applied the offline tracking method shown in (**Figure 3A**). While animal rotate the plus maze to choose lanes moving forward, backward and collecting reward, we notice the asymmetric movement of the two whisker during the most of behavior events, in particularly, during rotation (**Supplementary Video 10**).

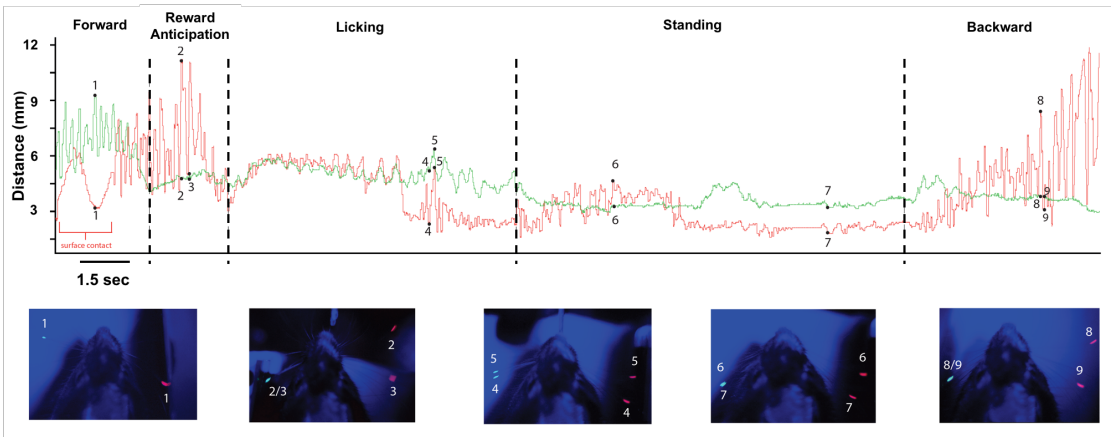

Figure 23. Combining Air-Track system with Pixy-Tracking system.

Tracking the movement of 2 whiskers (red on the right side and green on the left side of the face) on the Air-Track system. The two whiskers on the two side of whisker pad are almost invariably asymmetric in their motion. In the first epoch whiskers on one side move rhythmically, while the other side is in contact with a wall (forward movement of the animal in the track). During reward anticipation, the red whisker moves rhythmically, while the green whisker does not move at all. During licking behavior, the red whisker moves more. Even when the animal is just standing still, one whisker moves while the other stays almost still.

3.4. Discussion

This study demonstrates the utility of a color-tracking camera that can be used for rapid real-time tracking of two adjacent whiskers, limbs or even multiple animals. The method is flexible; it can work in various lighting conditions, it can be used for real-time data acquisition, and for automated tracking.

While earlier work in the whisker system has successfully used high-speed imaging, and electromyography to detect motion of the whisker pad or of individual whiskers, these methods have limitations. High-speed videography, one common approach (Arkley et al. 2014; Carvell and Simons 1990; Clack et al. 2012; Grant et al. 2009; Hartmann et al. 2003; Knutsen et al. 2005; Ritt et al. 2008; Sachdev et al. 2001; Voigts et al. 2015; Voigts et al. 2008), can have very high spatial-temporal resolution, but requires specialized algorithms. Another approach is to use electromyography (Berg and Kleinfeld 2003a; Carvell and Simons 1990; Fee et al. 1997; Sachdev et al. 2003; Zagha et al. 2013), which can report whiskers movement in real-time. However EMG is invasive and has insufficient spatial resolution. Alternatively, an array of sensors or a single laser / IR sensor can be used to track the location of a single whisker at a single point (Bermejo et al. 1996; O'Connor et al. 2013).

Some of these limits do not apply to Pixy cameras. The Pixy camera has a color-filtering algorithm built into it, that does not require the use of elaborate software or additional hardware. This camera can be used to track multiple body parts at the same time, and as a system, it is flexible enough to be rapidly reconfigured for monitoring any part of the body and / or even the whole animal. These cameras are designed to process data in real-time, and to track hues on any painted appendage; they can be used with a variety of objectives; they can be used to obtain real-time x, y and angle information of any colored object (Nashaat et al. 2016). An advantage of this system is that a painted object, even one that is slightly out of focus, can be tracked adequately.

Because Pixy uses color-based analysis, it can easily track multiple similarly shaped objects with ease and little confusion. It can work for almost any colored object tracking setting, with very little customized software. Another key advantage of Pixy over other systems, is that it is an open-source tool where almost every aspect of the process, from the Pixy data, Pixymon software, to the hardware i.e. objective used, to zoom, lighting, and coloring are accessible for modification.

A disadvantage of this system is that color is necessary and must be visible on the animal. Coloring the animal requires that animals tolerate the repeated application of body paint on their limbs or whiskers. Another disadvantage is that in real-time the maximum rate for Pixy cameras is only 50 Hz and can be slower (when used with USB input from Pixy instead of Arduino analogue waveform channels). For studies that require higher frequency movement (>~50 Hz), the Pixy camera can still be used to automatically track motion in slow motion videos. A major element of this experimental design is that the fast movements missed in real-time can be recaptured for analysis (due to the higher temporal resolution of high-speed cameras). Furthermore, key events (e.g. object contacts, etc.) can be still be tracked online using the Pixy camera during the behavior and can be used offline to quickly direct the researcher to important parts of the high-speed video images.

With these methods, it becomes possible to non-invasively, flexibly, and inexpensively configure experiments where motion or location of one or more whiskers, limbs, or even the movement of the animal is used as feedback to trigger rewards, optogenetic signals or even to change the real or virtual environment around the animal. While our methods are by no means the first using color filtering, the breadth of tracking – from ~ 10 micron thick whiskers to whole animals – is unique and makes our methods almost universally applicable, to a variety of settings and biological species (Bobrov et al. 2014; Cheung et al. 2014; Varga and Ritzmann 2016).

Chapter 4: Discussion

4.1. Open-source tools for neuroscience

Recent advances in computer science and software technology have helped the neuroscientists in the pursuit of relating neural activity to cognition (Devor et al. 2013). Novel tools that track behavior and precisely control the interaction of an animal/person with the external world have revolutionized our understanding of neural functions. Until recently, these advancements required sophisticated and complicated machines that required specialists in these technologies.

But more recent advance in information technology and changes in the nature of social networking have shifted the design of these equipment to a user-friendly level, making it accessible to the hobbyist and the layman (as well as non-specialist neuroscience laboratories). For instance, nowadays almost any smart phone is equipped with advanced tools such as high-speed high-definition camera, audio recording, gyroscope, accelerometer, GPS, etc. In addition, 3D printing technology and small microcontrollers such as Arduino and Raspberry Pi provide a platform for miniaturized packages of tools. These easy-to-build systems enable the younger generation of scientists to exploit and customize their experimental settings for system control and acquire data. Many of these systems come with control programs that are freely available over the web. The use of open-source tools in neuroscience has several advantages. 1) It provides an inexpensive and accessible platform that allows large-scale experimental control and data acquisition. 2) It enables experimental customization of technology based on different research questions and requires the minimum in expert knowledge. 3) It is flexible and easy to combine with virtually any behavioral or recording instrument.

Along the same line, many laboratories have developed their customized tools for recording, imaging or analyzing data (Freeman 2015). At the hardware level, studies have described a pedagogical way to build customized optical tools for imaging biological tissue (Wijnen et al. 2016).

Other studies focused on simple methods to construct a precise manipulators using 3D printed technology to enable better accessibility of imaging, recording setups to biological tissues (Campbell et al. 2014; Sharkey et al. 2016).

At the level of electronics, there are many laboratories that uses cheap microcontrollers such as Arduino or Raspberry pi to control behavioral system in animals and to perform mesoscopic optical imaging, multiunit electrophysiology or optical perturbation experiments in the brain (Murphy et al. 2016; Nguyen et al. 2016; Sanders and Kepecs 2014; Siegle et al. 2015).

At the software level, several laboratories have provided an open access source for their developed software on the GitHub platform, a web-based Git repository hosting service. These customized softwares can perform different computational tasks that includes analyzing large-scale data, constructing and controlling stimulus, and modeling neuronal activity (Freeman 2015).

In our research work, we have also developed a set of open-source tools to study brain function in a controlled quasi-natural environment. For both the Air-Track system and Pixy-Tracking systems, to build the system, we used tools such as the Arduino microcontroller, Pixy camera, combined with other sensors and motors used by hobbyist. With the advent of open-source software platforms such as Arduino and python, we can precisely control our stimulus paradigm and data acquisition. Although the initial phases of the construction and development were time consuming, we have been successful in investing for the long term as large-scale implementation and further customization of these methods becomes simple.

4.2. Methodological advancement

The studies shown in this thesis focused on approaches for investigating brain function in rodents during realistic behavior. This involved developing systems for combining state-of-the-art recording techniques with natural behavior as well as approaches for tracking and assessing the behavior itself. This kind of approach has been popular predominantly over the last decade or two of neuroscience. During the last two decades, technological developments in physics, chemistry and lately in digital technology were always mingled with a robust advance in our understanding of the relationship between the brain and behavior (Gomez et al; 2015; Devor et al., 2013). In particular, the last two decades has witnessed huge advances in optical techniques for exploring the brain, both recording and influencing neural activity. Two-photon imaging has made it possible to record from neurons in living tissue and this technique is now standard in many laboratories around the world (Denk et al. 1990; Helmchen and Denk 2005; Svoboda et al. 1997).

The Air-Track system presented here is ideally placed for use with two-photon imaging. Nevertheless, a similar system, the so-called “Mobile Home Cage” system, developed commercially by Neurotar LTD, uses a similar principle to us has combined it with 2-photon imaging (Kislin et al. 2014). In fact, most virtual reality systems are combined with 2-photon imagining and we expect no difficulties in future experiments with our system (Dombeck et al. 2010; Roth et al. 2016; Sheffield and Dombeck 2015). One of the disadvantages of most 2-photon systems is that the rodent is typically placed on a ball from which the animal is “perpetually falling” in the sense that the head is above the rest of the body (Dombeck and Reiser 2012; Minderer et al. 2016). The torque and other movement artifacts caused by this situation is reduced in our system because the animal is always flat on the Air-Track (Nashaat et al. 2016). We therefore expect that the implementation of 2-photon imaging in our system will be trivial.

In order to construct visual-created virtual environments using common air-ball systems, a sophisticated software is required for tracking the animal

and mapping these movements to the virtual world. Often imperfect mapping makes it difficult to estimate the perceptual experience of the animal, which is not necessarily intuitive for humans designing the mapping interface (Dombeck and Reiser 2012; Minderer et al. 2016). In Air-Track, there is no need for subjective experimenter input to map changes in the virtual world. Air-Track is a real-world physical device in which changes in the animal movement is necessarily reflected physically in the view of the animal. In addition, we have focused on the simple development of a simple system to make it very flexible with different designs of real environment. The Air-Track platform is made of 3D printed materials, which can be designed to have different types and structures of mazes based on the experimental design.

This advantage makes Air-track as comparable VR system where changing the animal environment is feasible based and the experimental design. While VR system construct a visually predominant world for the animal to navigate, Air-Track world is predominantly tactile in which three-dimensional objects constitute the world that animal navigates (Nashaat et al. 2016). For nocturnal animals that rely on olfactory and somatosensory modalities, Air-Track is advantageous in studying brain function in mice and rats by approaching their brain with its own relevant sensory terms.

In Air-Track system, we have shown complex behavior where mice navigate a plus maze performing multimodal discrimination go/no-go and 2AFC tasks. Mice perform a visual discrimination go/no-go task to choose a correct lane in a plus maze. Then, mice were advanced to a 2AFC discrimination task either to discriminate texture roughness or lane terminal aperture width. During Animal training on the Air-Track system, we can advance from mice performing simple task such as moving the platform and collecting reward to a multimodal contextual 2AFC discrimination task. Using real-time analysis of animal behavior, we managed to automatically systematize the shift between different levels of task complexity based on animal performance.

Although Air-Track system provides a unique platform to study complex behavior with high-precision electrophysiology and imaging tools, a tenacious

problem of relating micro-level behavior such as whiskers or limbs movement to neural activity still exists. Recently imaging and tracking software and hardware technology have provided a platform to quantitatively describe animal behavior (Gomez-Marin and Mainen 2016; Gomez-Marin et al. 2014). Earlier work to study whisker system kinematics on high-speed imaging, electromyography or IR beam sensors to detect motion of the whisker pad or of individual whiskers. Although these methods were successful in understand a body of information about animal behavior, it showed limitations. High-speed cameras require specialized customized algorithms that only work in particular setting. For instance, in the barrel research, many tracking software systems were developed to automatically track whisker movement combined with high-speed videography (Clack et al. 2012; Knutsen et al. 2005; O'Connor et al. 2010; Voigts et al. 2008). Although these automated systems have successfully managed to efficiently track whisker kinematics, the software complexity and using customized algorithms strict their use to very distinct behavioral conditions. This made these systems hard to copy and replicate by other laboratories without making substantial changes in the software or following the exact behavioral setting.

The Pixy-Tracking system enables flexible tracking and quantitative analysis of behavior in virtual any behavioral setting including the Air-Track system. This is an easy plug and play method for tracking multiple whiskers or both limbs in real-time at 50 Hz, in different light conditions. Using a color-based filtering algorithm expands the flexibility of the Pixy system to track different types of objects and body parts. In our experiment, we show that without any customization Pixy-Tracking system can monitor the movement of multiple whiskers, forelimbs, and head-direction. In addition, Pixy camera can be combined with high-speed videography in a post-hoc paradigm to automatically detect and track frames collected using high-speed cameras. This post-hoc pradigm expands the temporal resolution used by Pixy camera algorithm to a higher temporal resolutions decided by the limit of high-speed camera.

As the system is inexpensive, and flexible in monitoring different aspects of motion in animal, it expands the scope of behavioral analysis. In principle, this method can be used to provide feedback in real-time based on the position a distinct whisker or limb. As this system uses a color-filtering algorithm, it has major advantages over other whisker tracking algorithms. 1) It shows efficiency in tracking multiple distinct geometrically similar objects. 2) It is very flexible that requires almost no customization that could be virtually used in tracking any object at different settings. 3) It is an open-source tool that requires minimum computational adjustment that could develop in any biomedical laboratories.

By combing Air-Track system with Pixy-Tracking system, we substantially expand the dimensions of experimental possibilities within the behavioral science space by enabling high-level description and metric of complex behavior in head-fixed mice.

4.3. Future Directions

4.3.1. Neuroscience of the virtual versus the real world

The technological advancement in VR systems has progressively improved our understanding of complex cognitive process such as sensory-motor integration and spatial navigation. However, recent studies has used VR systems in comparison to real-world environment to study higher cognitive function and showed substantial variation in neural activity on the network level (Minderer et al. 2016).

Goal-directed navigation requires complex neural computation that entails integrating different sensory inputs to contribute to an abstract perception of space. As a high cognitive function, spatial navigation engages different cell types that are specifically tuned to different characteristics of the environment such as place cells, grid cells, and boarder cells. During real-world navigation a natural integration occurs between different sensory information such as visual, tactile, egocentric movement and vestibular input (Moser and Moser 2008; Rowland et al. 2016). However, in VR system, many elements of this integrative process are either completely missing such a vestibular and tactile inputs or virtually present such as visual input.

In VR system, the integration is represented mainly in the interaction between visual and motor modalities that are artificially simulated in a close-loop fashion that is often imperfect. Although different studies have recorded activity form place cells activity during VR navigation. The focus of these studies was biased to a limited population of place cells that its activity tuned by visual/motor integration, while missing the contribution of different cues that contributes to either the tuning of these cells or other set of neural population. Due to qualitative differences between the two environments, studies have shown that the place cells are differing tuned between virtual and real world (Acharya et al. 2016; Aghajan et al. 2015; Ravassard et al. 2013). One study showed that only 40% of place cells activated in real-world are active in virtual environment (Aghajan et al. 2015). Although VR system

has enhanced our understanding of neural basis of spatial navigation, it fails short to recreate the different constituents of the real-world experience.

To the contrary of other head-fixed behavioral system including virtual reality, Air-Track sytem is a technical advancement that brings the real-world experience to head-fixed preparations. Our results show that mice exhibit natural locomotor behavior during navigation and learn to discriminate multimodal stimuli. Despite lacking vestibular input due to head-fixed, Air-Track system provides all the constituents of the real-world including three dimensional physical space, boarders, etc. Also, it has a dynamic multimodal environment with optical and tactile flow that mimic burrow-like environment. In addition, as a real physical environment, the Air-Track avoids the caveat of imperfect mapping required in VR system that is often estimated by experimenter subjective experience.

4.3.2. Air-track for augmented reality neuroscience

VR systems had valuably contributed in understanding neural activity in human, and animals. A promising variation for VR systems, that gets back some sensory qualities of the real-world, had been developed recently and known as “Augmented Reality” (AR). In AR system, the subject views the real-world while navigating through a display connected to a motion tracking system such as head-mounted or a display. As the subject navigates the real-world, a computer generated virtual object are added to augment the real-world view. AR system has been used in many neuroscience and rehabilitation studies in addition to direct consumer game market, such as the location-based augmented reality game “Pokemon Go” (Chang et al. 2016; Chen et al. 2016; de Oliveira Roque 2016; Tarr and Warren 2002).

In animal research, AR system could be the right formula to study the real-world experience while maintaining the main advantages of VR systems. Air-Track could gear this type of experiments under electrophysiological investigation by mixing the real-world experiences with virtual visual stimulus. In addition to the mismatches in the sensory and motor mapping that VR

system provides, further complex multimodal mismatches could be introduced between the real and virtual world.

4.3.3. Real-time behavior for closed loop neuroscience

For decades, scientists have used to probe the brain with an open-loop approach using a linear reductionist method of “stimulus-to-response” manner (Kleinfeld et al. 2006). However, recent research has shown that the closed-loop neural network and feedback is part of almost every computational process occurs in the brain of behaving animals (Potter et al. 2014). Thus, with the increasing use of behaving animals in neuroscience, the use of closed-loop neuroscience experiments become an emerging trend in the design of many experimental paradigms (Ahissar and Assa 2016; Potter et al. 2014). During active sensing, animals interact with their environment in a relational and dynamic way that modifies further subsequent sensory experiences (Dombeck and Reiser 2012). The aim of closed-loop neuroscience is to introduce precise and well-controlled technologies to become part of these loops. These technologies can be used to precisely control and explore the functional dynamics of the interaction between neural networks and environment.

With Air-Track and Pixy-Tracking system, different level of closed-loop experiments can be combined with different neural recording and manipulation settings. Air-Track provides an indirect real-time report of animal movement on the macro-level, in addition to other information about the spatial orientation and discriminability. Pixy-Tracking system enables a direct behavioral repertoire of the micro movement of the animal including whisker kinematics and feet movement. With multi-level real-time behavioral report systems that can quantitatively dissect complex behavior, Air-Track and Pixy are powerful tools for applying closed-loop methods on brain network during natural behavior.

4.3.4. 24-hour approaches for head-fixed behavior

Head-fixed experiments in rodents hold some special problems even with the Air-Track system. Animals can typically be fixed to a head-post for

limited periods of time before they become tired or stressed. Even behavioural training in head-fixed animals requires a trained and qualified person to supervise the behavioural training sessions all the time, and one animal at a time. Since rodents, especially mice, take large numbers of trials to learn even simple tasks, it can take many weeks to train a single animal. In terms of manpower, this means that a typical scientific question requiring multiple mice in multiple tests can involve many man-months or even years of supervision.

A solution to this problem was addressed in recent study in which a voluntary head restraint system was combined with two-photon microscopy (Scott et al. 2013). The system enables cellular resolution functional imaging from different cortical regions during decision-making. In this system, rats were trained to voluntary restrain themselves for periods up to 8 seconds. The system mechanically automates repositioning the animal head in each trial of voluntary head-fixation to image the same neuronal population. Another study has built a similar system to perform a wide-field functional imaging in voluntary head-fixed mice (Murphy et al. 2016). This study was focused in developing a high-throughput automated voluntary head fixation system in home cage. Several mice can be trained in combination with 24-hour imaging of brain activity.

One of the main features of the Air-Track system is that the floating platform can be completely removed from the head-fixed system and deposited elsewhere, such as the home cage of the animals. This greatly alleviates the problem mentioned above, however for this purpose it is necessary to design an immobile version of the Air-Track system with rewards and tracking system that are analogous to the mobile version. These electronic control elements that we have added to the floating platform make it possible to place animals in the same real-world even when they are not head-fixed. This potential development could be used to train animals in both their home cage and on an Air-track when animals are head-fixed.

The key feature of this system is to use the platforms that are identical in shape, form, and cues to those used in the standard rat or mice cages. Animals will learn the task in their home cage, and consequently perform

better even when head-fixed. They will learn complex tasks faster, and show less evidence of stress when they are head-fixed. This system should fit into any standard mouse or rat cage, and can be designed into any multi animal housed enriched environment, where each animal's performance in a group can be tracked. The immobile coaching track is associated with electronics for reward delivery, electronics and optics for tracking animals in dark or light conditions, and this track can be associated with high speed imaging and rotary motors for turning, spinning, or moving the track, depending on the design of the air track used with head fixed animals. The complete system could be used to compare each animal's behavioural performance in the home cage overnight with the performance in the head fixed condition.

4.4. Ethical considerations

One of the major advantages of the approaches discussed in this thesis and the introduction of 24-hour recording strategies is that the stress of the animals can be reduced. Animals can be stressed by changes to their environment and so most *in vivo* recordings require a period of habituation for the animal (even without head-fixation) if the recordings are made in a new environment. These problems are alleviated considerably by home-cage experimentation and techniques such as voluntary head fixation. This is important on a number of levels, some of which rarely get mention in scientific publications. Of course, it is always preferable and often vital for the animals to be calm and not stressed for realistic behavior. This also applies to the firing of neurons during behavior. Another aspect of animal research is always the necessity to perform the study within ethical boundaries. While this is rarely a topic of the study itself, this issue particularly affects neuroscientists performing awake experiments. The strategies shown in this thesis are therefore important on this level as well.

Supplementary videos

Supplementary Video 1. A real-time video of mouse performing 2 full trials on Air-Track. The videos were collected in the dark at 30 Hz camera in infrared (IR) light, and played back in real-time. IR-light was switched on/off in the first few seconds to show that experiments were done near complete darkness.

Supplementary Video 2. A mouse is middle of the of Air-Track platform, rotating the platform around itself to choose a correct lane. The videos were collected in full light at 240 Hz, and played back in slow motion.

Supplementary Video 3. A mouse rotated the Air-Track platform around itself, and chose a correct, dark lane. At the end of the lane the reward actuator advanced down to the animal, and the mouse made the correct somatosensory discrimination and detected the rough walls. It licked the correct (the right spout) for reward. The videos were collected in full light at 240 Hz, and played back in slow motion.

Supplementary Video 4. A zoomed in view of a mouse in the Air-Track shows the whiskers touching the walls, and the forepaw and hindpaw hitting the ground. The videos were collected in full light at 240 Hz, and played back in slow motion.

Supplementary Video 5. Real-time tracking of D1 and D2 whiskers. Left panel shows the real-time data transmitted from Pixy to data files. The top right panel shows the simultaneously acquired high-speed video of the two whiskers, and the bottom right shows Pixy view. The D2 whisker is painted red, and shows up as the red waveform on the top left, the D1 whisker is painted green and is the green waveform on the left. The yellow/black boxes are the text mark indicators, showing that Pixy is transmitting data in real-time via the USB interface. The positions of the two whiskers do not overlap. They are not at the same point in space at the same time, in the videos or in the

waveforms. The set point of both whiskers changes from moment to moment (time 5 s in the video, to 8 s in the video). The actual distance moved in millimeters can be seen in both the high-speed and the Pixy video.

Supplementary Video 6. Pixy analysis of slow motion video data. The color high-speed video can be played back in slow motion (left panel), and Pixy camera and Pixymon (middle panel) can be used to track the position of the two whiskers and the data can be extracted into a data file (right panel).

Supplementary Video 7. Pixy in infrared illumination. A single painted whisker shown in the video on the right is tracked in real-time (left panel) with infrared illumination. At 3 seconds into the video the infrared light is turned off, and the tracking of the whisker stops as well. When the light is turned on again, the whisker can be tracked.

Supplementary Video 8. Pixy for tracking limbs. The painted limbs can be tracked in two dimensions (*x* and *y coordinates)*, Up/Down and side to side. The red traces on the left are the UP/Down and side to side movement of the left limbs. The green traces are for the right limb. The treadmill position and velocity are also shown in the traces below.

Supplementary Video 9. Tracking a single animal head rotation / direction and position in real-time. Pixy camera tracks a multi-colored piece of Styrofoam fixed on animal head-plate in regular light condition. The red traces on the top-left shows the angle of head-direction, while the blue traces in the middle-left and green trace in bottom-left shows the horizontal and vertical movement respectively.

Supplementary Video 10. Tracking two whiskers on Air-Track system using Pixy-Tracking system.

Abbreviations

VR	Virtual reality
LED	Light-Emitting Diode
fps	Frames per second
PC	Personal Computer
V1	Primary visual cortex
S1	Primary somatosensory cortex
ICSP	In-Circuit Serial Programming
NC	Normally Closed
G-N-G	Go/No-Go
2AFC	Two alternative-forced choice.
IR	Infrared.
s	Second
ms	Millisecond
KPa	Kilopascal
Psi	Pound-force per square inch
mm	Millimeter
cm	Centimeter
vs	Versus

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Publications

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Eigenständigkeitserklärung

Hiermit erkläre ich, die vorliegende Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben.

Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze einen entsprechenden Doktorgrad nicht.

Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin vom 27. Juni 2012.

Datum:

Unterschrift: